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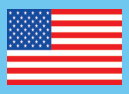
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Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
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Jian-Xia Cheng, Director
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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EDITORIAL

Role of taxanes in pancreatic cancer

Carmen Belli, Stefano Cereda, Michele Reni

Carmen Belli, Stefano Cereda, Michele Reni, Department of Oncology, San Raffaele Scientific Institute, 20132 Milan, Italy
Author contributions: Reni M conceived the paper and critically reviewed the data and the final version; Cereda S contributed to the analysis and interpretation of data; Belli C drafted the article and revised it critically for important intellectual content; and all authors approved the final version.
Correspondence to: Michele Reni, MD, Department of Oncology, San Raffaele Scientific Institute, via Olgettina 60, 20132 Milan, Italy. reni.michele@hsr.it
Telephone: +39-2-26437644 Fax: +39-2-26437625
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Abstract

Pancreatic cancer is one of the most deadly cancers and is characterized by a poor prognosis. Single agent gemcitabine, despite its limited activity and modest impact on disease outcome, is considered as the standard therapy in pancreatic cancer. Most of the combination regimens used in the treatment of this disease, also including the targeted agents, did not improve the outcome of patients. Also, taxanes have been tested as single agent and in combination chemotherapy, both in first line and as salvage chemotherapy, as another possible option for treating pancreatic cancer. The inclusion of taxanes in combination with gemcitabine as upfront therapy obtained promising results. Accordingly, taxanes, and above all, new generation taxanes, appear to be suitable candidates for further testing to assess their role against pancreatic cancer in various clinical settings.

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Key words: Pancreatic cancer; Advanced disease; Metastatic disease; Chemotherapy; Taxanes; Drug combinations; Radiotherapy; ABI-007

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INTRODUCTION

Pancreatic cancer has a very poor prognosis and it still remains as one of the most deadly cancers. At diagnosis, only 10%-20% of patients are considered candidates for a curative resection that is possible only in the absence of distant metastases, peritoneal carcinomatosis, and lack of any involvement of celiac axis and superior mesenteric artery[1]. Around 80% of patients have advanced or metastatic disease and median overall survival (mOS) of this group is very poor ranging from 3 to 6 mo[2]. The first drug used in the treatment of advanced pancreatic cancer was 5-fluorouracil (5-FU) that provided a palliative benefit with a significant improvement in mOS compared to best supportive care (6 mo *vs* 2.5 mo, $P < 0.01$)[3]. When compared to bolus 5-FU, gemcitabine yielded a significantly better response rate (RR) (5.4% *vs* 0%), survival (mOS 5.65 mo *vs* 4.41 mo, $P = 0.0025$) and clinical benefit, which is a composite measure consisting of reduction of pain, analgesic drugs intake and weight loss (23.8% *vs* 4.8%)[4]. Given the modest and disappointing impact on overall survival (OS) achieved with single agent gemcitabine, other agents or drug combinations are continuously tested in advanced pancreatic cancer[5-7]. Among others, taxanes (docetaxel and pacli-

taxel) were tested as single agents or in combination with other chemotherapeutic agents in pancreatic cancer[8-10] because they showed promising activity in other solid tumours[11,12]. Mechanism of action of these drugs consists in enhancing microtubule assembly and inhibiting the depolymerization of tubulin responsible for the formation of bundles of microtubules blocking cell proliferation[13].

This article summarises the main clinical studies conducted in pancreatic cancer with taxanes as first or second line chemotherapy, both as monotherapy and combination therapy, in order to clarify the potential role of this class of drugs for further investigations.

DOCETAXEL

First-line therapy

Single agent: Docetaxel gained researchers' attention for the treatment of pancreatic cancer after a preclinical study showed its effectiveness in a murine model of pancreatic ductal adenocarcinoma[14]. Docetaxel activity as single agent was assessed in phase Ⅱ trials in chemonaïve patients affected by pancreatic cancer at two different dosages, 60 mg/mq[8] and 100 mg/mq[9,15], showing promising activity when used at higher dose (Table 1). With higher doses[9,15], the overall RR ranged from 5% to 15%, the median time to progression (mTTP) was 2.1-5.0 mo, and the mOS was 7-8.5 mo. Neutropenia was the most frequently observed grade 3/4 toxicity in both studies (36%-95%)[9]. Grade 3-4 anemia (9%-16%) and fatigue (9%-23%) were also commonly reported[9]. Given the phase Ⅱ nature of the trials, the small sample size and the selection of patient population, including stage Ⅱ and Ⅲ disease and patients who mainly had a performance status of 0-1, these results should be interpreted with caution. Nevertheless, these trials showed that docetaxel has some activity in the treatment of pancreatic cancer, and warrant further exploration. Docetaxel administered at lower dose failed to demonstrate any activity in pancreatic cancer[8]. In fact, no objective response was observed and both mTTP and mOS were shorter when compared to higher doses[9,15]. Moreover, grade 3-4 toxicity was remarkable with nearly 80% of the patients developing neutropenia, 7% anaemia, and 27% fatigue[8].

Combination chemotherapy: Docetaxel role was also addressed in combination with other drugs (Table 1). *In vitro* and *in vivo* studies have demonstrated that docetaxel yields a synergism with other drugs like capecitabine[16,17], 5-FU[18], gemcitabine[19,20], and cisplatin[21] .

The rationale for the combination of an oral fluoropyrimidine with docetaxel is based on the ability of taxanes to increase the activity in the tumoral tissue of thymidylate phosphorylases, which are key enzymes in the transformation process of capecitabine into its active metabolite 5-FU. The synergism of docetaxel with gemcitabine was observed *in vitro* in several cancer cell lines, but the biological mechanism is not clear. Hypothetically, the combination of these two agents could regulate the apoptotic process by increasing the apoptotic index. Finally, the synergism of docetaxel and cisplatin, observed in cell lines of gastric cancer, could be due to the down-regulation of multi drug resistant proteins by docetaxel thereby increasing cytotoxic index of cisplatin[21].

Several phase Ⅱ clinical trials assessed the activity of these combinations against advanced pancreatic cancer as an upfront therapy[19,20,22-26]. Docetaxel and gemcitabine yielded promising RR ranging from 12% to 40% and mOS ranging from 6 to 9 mo[19,20,23,25]. However, these single arm findings cannot be considered conclusive. Interestingly, the European Organization for Research and Treatment of Cancer (EORTC) group conducted a phase Ⅱ trial in which 96 patients were randomized to receive gemcitabine plus docetaxel or cisplatin plus docetaxel[24]. In 70 patients who were assessable for response, the RR was 19.4% with gemcitabine-docetaxel combination and 23.5% with cisplatin-docetaxel combination. Conversely, survival figures were better in 49 patients treated by the gemcitabine-docetaxel combination [median progression free survival (mPFS) 3.9 mo, mOS 7.4 mo; 1-year OS 30%] compared to those receiving cisplatin-docetaxel (mPFS 2.8 mo, mOS 7.1 mo; 1-year OS 16%). Toxicity was not negligible, consisting of grade 3-4 neutropenia in 47% and 55%, and febrile neutropenia in 9% and 16%, respectively. Altogether, safety profile and survival analysis favoured gemcitabine-docetaxel combination for further evaluation. Another phase Ⅱ trial randomized 259 patients with metastatic pancreatic cancer to receive fixed dose gemcitabine or gemcitabine combined with either docetaxel, cisplatin, or irinotecan[27]. The primary end point of this study was the six months OS that was similar in all four treatment arms: 57% for fixed gemcitabine dose, 53% for gemcitabine plus cisplatin, 54% for gemcitabine plus docetaxel, and 57% for gemcitabine plus irinotecan. The mOS and mTTP were similar in the treatment groups and ranged between 6.4-7.1 mo and 3.3-4.5 mo, respectively. The RRs were also indistinguishable among treatment groups and ranged between 12% to 14%. The cisplatin and docetaxel combination tested in this study gave similar results, in terms of mOS and TTP, to that reported in the EORTC trial[24].

Another drug tested in combination with docetaxel was liposomal doxorubicin starting from preclinical study conducted on xenografted human pancreatic carcinoma in which this drug demonstrated to reduce tumor growth with a low toxicity[28]. In a phase Ⅱ clinical trial, this combination was studied on twenty-one locally advanced and metastatic pancreatic cancer patients[29]. The results in terms of RR (21%) and mOS (10 mo) were similar to those observed with combination of docetaxel with other drugs[19,20,22-26].

The activity of docetaxel, gemcitabine, capecitabine regimen (GTX) was also tested in 43 patients with metastatic disease yielding a RR of 22% and a mOS of 14.5 mo[30]. These results were echoed in a recent retrospective study on 79 chemonaïve patients with locally advanced or metastatic pancreatic cancer who had a mOS

Table 1 Clinical trials of docetaxel in pancreatic cancer

Trial	CT agent	Line CT	No. of patients	RR %	mPFS (mo)	mOS (mo)	Toxicity %
Okada *et al*[8]	Doce	I	21	CR 0 PR 0 SD 33	1[1]	6	Neutropenia 86, anemia 10, thrombocytopenia 5, asthenia 33, nausea-vomiting 29
Androulakis *et al*[9]	Doce	I	33	CR 3 PR 3 SD 58	5[1]	8.5	Neutropenia 36, febrile neutropenia 6, anemia 9, asthenia 9, neuropathy 6
Rougier *et al*[15]	Doce	I	43	CR 0 PR 15 SD 3	2.1[1]	7	Neutropenia 95, febrile neutropenia 9, anemia 16, asthenia 23, vomiting 7
Stathopoulos *et al*[23]	Doce + GEM	I	54	CR 0 PR 13 SD 33	8[1]	6	Neutropenia 31, febrile neutropenia 11, thrombocytopenia 7, asthenia 13, diarrhea 6
Ryan *et al*[25]	Doce + GEM	I	33	CR 0 PR 18 SD 39	3.8	8.9	Neutropenia 49, febrile neutropenia 12, asthenia 27, nausea-vomiting 12, diarrhea 12, neuropathy 9
Lutz *et al*[24]	Doce + GEM Doce + CDDP	I	96	CR 0/2.9 PR 19.4/20.6 SD 36.1/35.3	3.9/2.8	7.4/7.1	Neutropenia 40/50, febrile neutropenia 9/16, anemia 20/9, thrombocytopenia 8/5, diarrhea 8/5, stomatitis 8/10
Kulke *et al*[27]	GEM + CDDP GEM FDR GEM + Doce GEM + CPT-11	I	259	CR 2/0/0/2 PR 11/14/12/12 SD 54/58/53/55	4.5/3.3/4.1/4.0	6.7/6.4/6.4/7.1	Neutropenia 46/48/31/25, febrile neutropenia 2/3/5/2, anemia 16/12/16/5, thrombocytopenia 49/25/9/14, asthenia 16/14/21/19, nausea-vomiting 41/26/17/25, diarrhea 80/2/8/8
Fine *et al*[30]	GTX	I	43	CR 0 PR 21.9 SD 41.5	6.9[1]	14.5	Neutropenia 29.2, thrombocytopenia 12.2, mucositis 7.5
Reni *et al*[22]	PDXG PEXG	I	105	CR 2/4 PR 58/33 SD 19/46	7.4/7.6	10.7/11	Neutropenia 4/13, thrombocytopenia 2/4, anemia 4/4, asthenia 6/3
Cereda *et al*[35]	Doce	II	10	CR 0 PR 0 SD 20	1.5	4	Not observed
Katopodis *et al*[37]	Doce + X	II	31	CR 0 PR 9.7 SD 22.6	2.4	6.3	Neutropenia 32.2, febrile neutropenia 3.2, anemia 3.2, thrombocytopenia 3.2, stomatitis 3.2, asthenia 6.5
Reni *et al*[39]	MDI	II-III	15	CR 0 PR 0 SD 20	1.7	6.1	Phase I study Neutropenia 23, fatigue, diarrhea, and vomiting 10

[1]mTTP: Median time to progression; CT: Chemotherapy; RR: Response rate; mPFS: Median progression free survival; mOS: Median overall survival; Doce: Docetaxel; GEM: Gemcitabine; CDDP: Cisplatin; CPT-11: Irinotecan; GTX: Gemcitabine + Taxotere + Xeloda; PDXG: Cisplatin + Docetaxel + Gemcitabine + Xeloda; PEXG: Cisplatin + Epirubicin + Xeloda + Gemcitabine; X: Xeloda; MDI: Mitomycin + Docetaxel + Irinotecan; CR: Complete response; PR: Partial response; SD: Stable disease.

of 25.0 and 11.3 mo, respectively[31].

A four drug combination of cisplatin, docetaxel, capecitabine, and gemcitabine (PDXG) was tested in a randomized phase II trial in which a cisplatin, epirubicin, capecitabine, and gemcitabine (PEXG) regimen was chosen as calibration arm[22]. This choice was based on the fact that a PEFG regimen (cisplatin, epirubicin, fluorouracil, and gemcitabine) was previously shown to be superior to gemcitabine monochemotherapy in terms of progression free survival [PFS; hazard ratio (HR) 0.51; range 0.33-0.78] and OS (HR 0.65; range 0.43-0.99) in a phase III trial of first line therapy of pancreatic cancer[32] and that the use of oral capecitabine was shown to be equivalent to 5-FU in other tumors[33]. Both the radiological and the biochemical RR[34] were better for 53 patients treated with PDXG (60% complete plus partial radiological responses; 41% major biochemical responses; 39% minor biochemical responses) than for 52 patients receiving PEXG (37% complete plus partial radiological responses; 32% major biochemical responses; 32% minor biochemical responses). However, OS and PFS were very similar in the two arms (mOS 10.7 mo *vs* 11.0 mo and mPFS 7.4 mo *vs* 7.6 mo, with PDXG and PEXG regimens, respectively). The safety profile of PDXG regimen was more favourable than that of PEXG regimen in terms of grade 3-4 neutropenia (4% in PDXG group *vs* 13% in PEXG arm).

Overall, these studies suggest that multi-drug associations, in particular triplets and quadruplets, are more active in pancreatic cancer when compared to monochemotherapy.

Salvage therapy

Single agent and combination chemotherapy: Docetaxel was also tested as salvage treatment in pancreatic cancer both as single agent and in combination[31,35-40]

Table 2 Clinical trials of paclitaxel as single agent or in combination chemotherapy in pancreatic cancer

Trial	CT agent	Line CT	No. of patients	RR %	mPFS (mo)	mOS (mo)	Toxicity %
Whitehead *et al*[10]	PTX	I	45	CR 3 PR 5 SD 13	NR	5	Neutropenia + leukopenia 92 , anemia 23, thrombocytopenia 20, asthenia 23, nausea-vomiting 18, neuropathy 7
Saif *et al*[46]	GPM	I	56	CR 1.7 PR 3.5 SD 54.8	2.8	6.5	Neutropenia 40, asthenia 17.8, neuropathy 13.3
Löhr *et al*[48]	GEM GEM + ET 11 mg/mq GEM + ET 22 mg/mq GEM + ET 44 mg/mq	I	212	CR 0/0/0/0 PR 14/14/14/16 SD 30/46/51/35	2.7/4.1/4.6/4.4	6.8/8.1/8.7/9.3	Neutropenia 18/12/16/22, anemia 4/0/4/8, thrombocytopenia 2/8/16/14, nausea + vomiting 2/2/0/10
Von Hoff *et al*[50]	Nab-PTX + GEM	I	67	CR 4 PR 42 SD 18	7.9	12.2	Neutropenia 67, thrombocytopenia 23, asthenia 21, neuropathy 15
Hosein *et al*[51]	Nab-PTX	II	19	CR 0 PR 5.3 SD 31.6	1.6	7.3	Neutropenia 32, febrile neutropenia 11, anemia 11
Kim *et al*[52]	PTX + 5-FU	II	28	CR 0 PR 10 SD 20	2.5[1]	7.6	Leukopenia 6, diarrhea 2, neuropathy 1

[1]mTTP: Median time to progression; CT: Chemotherapy; RR: Response rate; mPFS: Median progression free survival; mOS: Median overall survival; PTX: Paclitaxel; NR; Not reported; GPM: Paclitaxel loaded polymeric micelle, ET: Paclitaxel embedded in cationic liposomes; Nab-PTX: Paclitaxel protein-bound particles; 5-FU: 5-fluorouracil.

(Table 1). In a phase II trial conducted on 10 patients, no response was obtained, mPFS was 1.5 mo and mOS was 4.0 mo[35].

Combination chemotherapy with taxanes as salvage treatment gave disappointing results with a significant toxicity[37-39]. In these trials, docetaxel combined with capecitabine[37], with irinotecan[38], and with mitomycin plus irinotecan[39] resulted in a RR ranging between 0% to 9.7%, and mOS between 4.5-6.3 mo. The most common toxicity in these studies was grade 3-4 neutropenia observed in around 30%-32% of patients[37-39]. Two retrospective series reported the results of GTX regimen as salvage therapy in patients affected by pancreatic cancer[31,40]. The RR was 12%-15% and mOS was 5.7-6.7 mo[31,40]. Altogether, the response and survival figures observed with docetaxel-based combinations as salvage therapy in advanced pancreatic cancer were in the range reported with other regimens[41-45]. These drug combinations tested in the second line gave RR between 0%-24% and mOS between 3.7-6.2 mo[41,42,44,45]. The PEFG combination was the only regimen that reported a better mOS in second line therapy (8.3 mo) with an acceptable toxicity[43].

PACLITAXEL AND NEW PACLITAXEL FORMULATIONS

First-line therapy

Single agent: Single agent paclitaxel yielded a RR of 3% and a mOS of 5 mo in a series of 45 patients with advanced pancreatic cancer (Table 2)[10], while paclitaxel loaded with polymeric micelle obtained an overall RR of 6.7%, mPFS of 2.8 mo, and mOS of 6.5 mo in 56 patients with advanced pancreatic cancer[46].

Combination chemotherapy: EndoTAG™-1 (ET) is a cationic liposome membrane charging paclitaxel. This particular structure promotes the delivery of the drug in the tumor mass. Tumor endothelium lacks glycocalix which normally covers endothelial cells, so negative charges are exposed on the cell surface. Thus, the positive charges carried by liposomes is exposed and interact with the negative charges present on tumoral cells favouring the internalization of the drug into the tumor[47].

Löhr *et al*[48] tested ET in combination with gemcitabine *vs* gemcitabine alone in a four-arm randomized phase II trial on 212 patients affected by locally advanced or metastatic disease (Table 2). The treatment consisted of seven weekly infusions of standard gemcitabine alone or associated with twice-weekly ET at dosage of 11 (Endo11), 22 (Endo22) or 44 mg/mq (Endo44) for seven weeks. RR was comparable across the four treatment groups (14%-16%), the mPFS was longer in the gemcitabine plus ET arms (4.1, 4.6, and 4.4 for Endo11, Endo22, and Endo44, respectively) compared to gemcitabine group (2.7 mo). Also the mOS appeared to be better in the combination arms (from 8.1 to 9.3 mo) compared to single agent (mOS 6.8 mo). The treatment with gemcitabine and ET was well tolerated with a dose-dependent increase in grade 3-4 thrombocytopenia (from 8% to 14%), neutropenia (from 12% to 22%), and anemia (from 4% to 8%). Grade 3-4 febrile neutropenia was observed in 6% of the patients. No treatment-related neuropathy was observed in this trial. This study suggested that this new formulation of paclitaxel warrants further investigation to define its role in the treatment of pancreatic cancer.

Another paclitaxel formulation known as ABI-007 was tested against pancreatic cancer. ABI-007 (also

known as *nab*-paclitaxel), is a cremophor-free, albumin-bound 130-nm particle form of paclitaxel that does not require the use of cremophor-EL, thus avoiding the severe toxicities associated with this vehicle[49]. The albumin in nab-paclitaxel binds to gp60 (albondin) receptors and to caveolae resulting in the formation of caveoli transporting the drug across the endothelial cells to the tumor interstitial space. In pancreatic tumor stroma, secreted protein acid and rich in cystein (SPARC) protein, which is also called osteonectin, is overexpressed. SPARC interacts with the albumin of nab-paclitaxel enhancing the concentration of this drug into the tumor, which causes "stromal collapse", a phenomenon of depletion and collapsing of stroma, bringing tumor cells closer to each other and to blood vessel. A phase IB-Ⅱ study of ABI-007 in combination with gemcitabine was performed in metastatic pancreatic cancer (Table 2)[50]. The maximum tolerated dose (MTD) was 125 mg/mq for ABI-007 in combination with standard gemcitabine[50]. The PFS for the whole population of patients enrolled into the trial was 6.9 mo and the mOS was 10.3 mo, while in the group of 44 patients treated with ABI-007 at MTD, the mPFS was 7.9 mo and the mOS was 12.2 mo. A phase Ⅲ clinical trial of gemcitabine and ABI-007 combination *vs* standard gemcitabine is currently ongoing in patients with metastatic pancreatic cancer (NCT00844649).

Salvage therapy

Single agent and combination chemotherapy: ABI-007 at 100 mg/mq weekly for three weeks, out of every 4, was also tested as second line chemotherapy in 19 patients with progressive pancreatic cancer after previous gemcitabine-based therapy (Table 2)[51]. One partial response (5.3%) and six stable disease (31.6%) were reported. The mPFS and mOS were 1.6 mo and 7.3 mo, respectively. Grade 3 or 4 neutropenia, neutropenic fever and anemia occurred in 32%, 11% and 11% of patients, respectively.

Paclitaxel in combination with 5-FU was administered as salvage therapy to 28 patients with advanced pancreatic cancer after gemcitabine failure (Table 2)[52]. The RR was 10%, the mTTP 2.5 mo, and the mOS 7.6 mo. This regimen was well tolerated with grade 3-4 neutropenia in 21.4% of the patients, anemia in 3.6%, grade 4 neuropathy in 3.6%, and grade 3 diarrhea in 7.2% of the patients.

TAXANES PLUS RADIOTHERAPY

Pancreatic cancer is characterized by a high rate of both local and systemic failure. Chemoradiation was tested in stage Ⅲ disease with different drugs yielding a mOS between 8 to 11 mo and 1-year survival rate between 25% to 40%[53-55].

Due to their radiation-sensitizing properties[56], taxanes were also tested in combination with radiotherapy in locally advanced[57,58] and in resectable[59] pancreatic cancer. In locally advanced disease, paclitaxel and radiotherapy obtained RR of 26%, mOS of 8-11.2 mo and 1-year OS of 30%-43%[57,58]. These results were in the range reported with other drugs tested in combination with radiotherapy.

Conversely, paclitaxel-based chemoradiation as neoadjuvant therapy in resectable patients yielded disappointing results[59]. In fact, 46% of the patients suffered grade 3 toxicity (hematological, gastrointestinal, asthenia, anorexia, allergic reaction) and the mOS (19 mo) was inferior than expected with 5-FU based chemoradiation (25 mo)[60].

A phase Ⅱ trial randomized 20 patients with resectable and unresectable disease to receive docetaxel plus either continuous 5-FU or weekly cisplatin concomitant to radiotherapy. The enrolment was prematurely concluded due to poor preliminary results[61].

CONCLUSION

Pancreatic cancer is characterized by a dismal prognosis and limited therapeutic progress has been achieved in the past 30 years. Due to its intrinsic or rapidly acquired chemoresistance, the therapeutic armamentarium against pancreatic cancer is limited and there is an urgent need to individuate new active agents or regimens. Single agent gemcitabine, despite poor activity and modest impact on disease outcome, is still considered the standard treatment both in early and advanced stages of the disease[62]. Most combination regimens using gemcitabine-based doublets and including both conventional and targeted agents failed to significantly improve OS over gemcitabine alone[6,7,33,63,64] or yielded a statistically significant but clinically negligible benefit[65]. Interestingly, two phase Ⅲ trials showed that drug combinations including more than two agents may improve OS when compared with gemcitabine alone[32,66] and two clinical practice surveys suggested that the 4-drug regimens may be superior to gemcitabine/platinating-agent doublets[41,67].

In particular, the PEFG regimen and the combination of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) yielded 1-year OS of 38%-48%[32,66]. The results obtained with these regimens should be generalized with caution due to the lack of confirmatory trials and, in the case of FOLFIRINOX, to the highly selected patients population, which is evident on the basis of the better than expected standard arm outcome and because 4 years occurred to enrol 342 patients in 48 centers (< 2 pts/center per year)[66]. Moreover, while PEFG toxicity profile was favourable[32,68], and the regimen was in fact feasible also in the adjuvant setting[69,70], grade 3-4 toxicity observed with FOLFIRINOX was remarkable particularly in the case of extra-hematological toxicity that may be barely acceptable in the context of a palliative therapy. In fact, the main reason for ending treatment was death in 85 (50%) patients in the FOLFIRINOX arm *vs* 75 in the gemcitabine arm, while fatigue was reported in 24% of the patients, vomiting in 15%, diarrhea in 13%, and neuropathy in 9%[66]. Altogether, these results are encouraging and do suggest that a nihilistic attitude towards pancreatic cancer is no

longer justified and that more aggressive treatment approach may partially overcome chemoresistance. As previously observed with other drugs, like gemcitabine, 5-FU, capecitabine, pemetrexed[4,6,71], the use of taxanes as single agent treatment, both upfront and as salvage therapy, showed moderate activity but did not obtain exciting results[8,9,15,35,36]. Not surprisingly and similarly to fluoropyrimidines and platinating agents, the inclusion of old generation taxanes in doublets with gemcitabine or cisplatin did not appear to produce better results than gemcitabine alone[19,20,23-27,29,37-39]. On the other hand, the inclusion of taxanes in combination with more than 2 drugs[22,30,31,40] seem to be more promising. Worth of note, unexpected radiological and biochemical response was observed in an exploratory subset analysis in patients with stage Ⅲ disease (60% radiological response in PDXG group *vs* 37% in PEXG group and major plus minor biochemical response of 80% *vs* 64%, respectively[22]). Furthermore, more patients in PDXG arm underwent to surgery with radical intent compared to PEXG arm (17% *vs* 6%) and neither resection margin nor nodal involvement was observed in the group treated with docetaxel. Furthermore, the new generation of taxanes, due to their unique chemical structure, are able to penetrate in tumor cell mass in high amount and apparently yields better activity than older taxanes. Accordingly, taxanes, and above all, new generation taxanes, appear to be suitable candidates for further testing to assess their role against pancreatic cancer in various clinical settings.

FUTURE PERSPECTIVES

Apart from the combination of ABI-007 with gemcitabine as first-line therapy in metastatic disease, which is currently being tested in a phase Ⅲ trial (NCT00844649), the role of multiple (i.e., more than two drugs) agents regimens should be addressed. In fact, the hypothesis of stromal depletion induced by ABI-007, if confirmed, may provide a robust rationale for combination polychemotherapy, due to better drug penetration into tumor. Furthermore, a larger effect may be expected in primary tumor where the stroma is more abundant[72-74]. A phase Ⅱ clinical trial is evaluating a combination of ABI-007 with gemcitabine, and GDC-0449, a hedgehog inhibitor, in patients with untreated metastatic pancreatic cancer in order to evaluate the PFS and the safety of this combination (NCT01088815). The hedgehog signalling pathway is involved in embryonic development, but is also activated in pancreatic cancer[75]. In preclinical model the inhibition of this pathway enhanced drug delivery to tumor cells by disrupting the desmoplastic stroma and increasing tumor vascularity[76]. The combination of gemcitabine, ABI-007 and GDC-009 could enhance the stroma collapse and increase the intratumoral concentration of chemotherapeutic drugs. Accordingly, neoadjuvant therapy in patients with stage Ⅲ disease, borderline resectable disease and resectable disease represents a potential field of investigation. Finally, ABI-007 may improve primary tumor oxygenation by inhibiting the formation of novel microvessel and by disrupting established microvessels thus increasing the therapeutic window of concomitant radiation therapy and targeted agents[77,78]. So, the next logical step is to evaluate a combination of anti-angiogenic therapy with ABI-007 in metastatic setting. Furthermore, the identification of new prognostic markers like SPARC could help both in understanding the molecular changes responsible for development and progression of pancreatic cancer and in identifying a subset of patients in which taxane-based therapy may have a more relevant impact on the outcome.

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FIELD OF VISION

Confounding factors affect the pathophysiology of eosinophilic esophagitis

Yoram Elitsur

Yoram Elitsur, Department of Pediatrics, Gastroenterology Division, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25701, United States
Author contributions: Elitsur Y solely contributed to this manuscript.
Correspondence to: Yoram Elitsur, MD, Professor in Pediatrics, Department of Pediatrics, Gastroenterology Division, Joan C. Edwards School of Medicine, Marshall University, 1600 Medical Center Drive, Huntington, WV 25701, United States. elitsur@marshall.edu
Telephone: +1-304-6911300 Fax: +1-304-6911375
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Abstract

Eosinophilic esophagitis is a newly diagnosed esophageal disease in adult and children. The clinical and pathological characteristics of this disease have been established and were recently summarized in the expert clinical guideline published in 2011. In spite of the wide knowledge accumulated on this disease, there are many areas where scientific data are missing, especially in regard to the disease's pathophysiology. Recent publications have suggested that other confounding factors modify the disease and may affect its clinical-phenotypic presentation. Those factors may include place of living, air pollution, race, genetic factors and other. In the present report we discussed and review those confounding factors, the new developments, and what direction we should go to further advance our knowledge of this disease.

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Key words: Eosinophilic esophagitis; Confounding factors; Race; Gender; Environment

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INVITED COMMENTARY ON HOT ARTICLES

Eosinophilic esophagitis (EoE) is a chronic esophageal disease associated with allergy in adults and children. The clinical, endoscopic, and histological characteristics of EoE has been described and documented in several expert reports[1,2]. In spite of the knowledge accumulated on this newly discovered disease, double blind placebo control studies have demonstrated that only about 50% of the patients will respond to therapy (biologic, steroids)[3,4], demonstrating the existing gap between the clinical and/or basic knowledge, proposed therapy and the pathophysiology of EoE. The complete story of EoE in children is yet to be unfolded.

In a recent publication, Sperry *et al*[5] compared the clinical presentation of EoE in a cohort of adult and pediatric patients diagnosed with EoE. The authors suggested that African American (AA) patients were younger at diagnosis, presented with failure to thrive (FTT), and were less likely to have esophageal rings compared to the Caucasian (C) patients[5]. The paper has raised the possibility of other unknown confounding factors that may influence the clinical/phenotypic presentation of EoE in adults and children. Indeed, reviewing the

literature of EoE disease suggested that various factors may modify the disease incidence, clinical presentation, and/or clinical response to therapy. Those possible confounding factors are the topic of this paper (Table 1).

Epidemiological studies have showed that the disease's rate is unevenly distributed among adults and children living across the United States. The data showed that the prevalence of the disease in the North-Eastern states is higher in comparison with the South-Eastern states[6-8]. Moreover, further analysis demonstrated that EoE is more prevalent in urban/sub urban areas compared to rural areas[6,8,9]. The data suggested that there are environmental factors that modify EoE, but to date, those modifiers have not been clearly analyzed. The current geographic distribution may implicate air pollution as a modifier factor for EoE[10]. Indeed, animal and clinical studies have confirmed the essential role of aero-allergens in the pathophysiology of the disease[11-13]. Few investigators reported an increase incidence of EoE during the allergy seasons (low during winter, high during spring and fall)[14-16], but those findings were not confirmed by others. For example, in a recent preliminary report, Frederickson *et al*[17] reviewed esophageal biopsies in a cohort of 19 172 patients in Iowa City, IA, of whom 167 adults had EoE. The author reported a comparable monthly rate of EoE with no seasonal variation. Using a national pathology laboratory data base, the seasonal trend of 9995 adult patients was examined by others who reported a similar monthly and seasonal distribution of the patients diagnosed with EoE[18]. Similar results were described by investigators who performed an internet search to look for EoE and seasonal variation[19]. In a recent study, Hurrell *et al*[20] reported a higher prevalence of esophageal eosinophilia in adults who live within the colder geographic zones of the United States compared to those living in the warmer climates. They implicated that the specific flora grow in those areas generate highly potent aeroallergens, and is responsible for their findings. In contrast, the United States governmental environmental agency (Environmental Protection Agency, National Oceanic and Atmospheric Administration, National Park Service) reported the lowest air pollution in the northern geographic areas of the United States, those with colder climate rather than the warmer climate (http//airnow.gov).

In addition to the possible role of aeroallergens and pollution in the pathophysiology of EoE, clinical reports have documented the etiologic role of food allergy in this disease[3,11,21-23]. Indeed, the updated clinical guideline recommended checking food allergy in any patient who is newly diagnosed with EoE[1]. Moreover, in support of this role, eliminating the food allergens alone (i.e., hypoallergenic formula) resolved the clinical symptoms and reversed the histological changes typically seen in EoE[1,24-26]. In patients that would not accept this mode of therapy, topical or systemic steroids are suggested[1]. In a randomized, double-blind, placebo control study, EoE children were treated with Fluticasone propionate[3].

Table 1 Confounding factors and eosinophilic esophagitis

Factor	Ref.
Geographic distribution	[6-9,20]
Air pollution (aeroallergens)	[11-13]
Food allergy	[21-23]
Race (ethnicity)	[27-35]
Gender	[21,26]
Genetic factors	[22,23,39,40]

The authors showed that the children with no allergy had a better response to therapy compared to the children with allergy, pointing towards the role of food allergens as modifiers[3]. Indeed, the role of food allergens in the pathophysiology of EoE has been established, but we are still lacking the exact function of these modifiers. Are all food allergens equal? What about the EoE patients who tested negative for any allergy? Is their disease is more benign? Do they achieve symptom control easier? All these questions are still unanswered and need further investigation.

The different distribution of EoE among various ethnicities has been previously published in adults and children. For example, the clinical presentation of EoE in children reported from several United States centers showed a statistically higher disease rate in C compared to AA or hispanic[21,27-29]. Other reported similar findings in the adult population[5,30,31]. This discrepancy is further accentuated by the epidemiologic data showing that in spite of the wide global distribution of EoE, very few reports from the African continent or from the east Asian countries were reported[1,32-34]. In recent years we have assessed the different clinical presentation of EoE between AA and C children. In that study, we compared between AA children living in urban neighborhoods in New York City and C children who are living in rural West Virginia[35]. We reported that EoE in AA children presented at an earlier age, have more history of atopic features, and presented with significantly lower endoscopic features of EoE compared to the C children. Similarly, Sharma *et al*[36] reported that AA children are more likely to present with FTT, gastroesophageal reflux disease symptoms, and at a younger age compared to the C children at the same cohort. Sperry *et al*[5] compared the clinical presentation of EoE in a cohort of adult and pediatric patients diagnosed with EoE. The authors suggested that when compared with C children, AA children diagnosed at a younger age, present with FTT, and have less esophageal rings[5].

Overall, these data suggest that racial factor does play a role in EoE disease and may serve as a modifier in the disease phenotypic presentation. Further studies will be needed to fully establish the role of ethnicity in the pathophysiology of EoE.

Male sex is the predominant gender in EoE[1]. In large series, the male/female ratio was reported up to 3:1[21,37]. The effect of gender on EoE disease has not been well investigated and studies on this subject are

lacking. Rothenberg *et al*[38] suggested that the predominance of males in EoE may be related to the thymic stromal lymphopoietin receptor resided within the pseudoautosomal region 1 on the X and Y chromosomes (Xp22.3, Yp11.3). The author suggested that this region may explain the higher incident of male gender in EoE.

Similar to many other chronic diseases; i.e., inflammatory bowel disease, celiac disease, asthma, *etc*., genetic factors have a significant influence on the prognosis of EoE. Indeed, several investigators evaluated the genetic factors related to EoE. Few investigators reported that EoE tends to cluster in families[22,23], and other showed by genome-wide microarray expression analysis that EoE patients have a different spectrum of genes compared to children with gastroesophageal reflux disease or normal control[39]. Others showed that specific genes are closely associated with eosinophil's activation and are increased in patients with EoE[1,4,39,40]. In another study, Lu *et al*[41] identified 32 different miRNA which are specific for EoE, of which miRNA-21 and miRNA-223 were the most up-regulated, and decreased post steroid therapy. In a preliminary report, Gonsalves *et al*[42] reported that the esophageal epithelial barrier genes (Desmoglein 1) is lower in adults with EoE who did not respond to dietary therapy, suggesting a dysfunctional, leaking esophageal mucosa. The new data may explain the role of aeroallergens in the disease's pathophysiology, by permitting the absorption of allergens (aeroallergens or food allergens) through the tightly sealed epithelial layer of the esophagus, and/or may explain the quick absorption of topical steroid into the esophageal mucosa during therapy.

Overall, the genetic framework involved in the pathophysiology of EoE is emerging and the genes responsible for activation or suppression of the inflammatory process are slowly uncovered. Nevertheless, we still have a large gap of knowledge left to explain the different response to therapy in EoE patients who belong to a different gender, different ethnicity, or have different allergies.

In summary, eosinophilic esophagitis is a chronic disease of the esophagus with clinical and histological characteristics previously established and described[1]. As in many chronic diseases, the clinical and/or histological presentation may be altered by various environmental or genetic modifiers. Epidemiological and clinical reports have documented the different phenotypic presentation of EoE in different populations, but further investigation is needed to dissect those disease modifiers in order to be able to tailor therapy for the specific patient. In the era of personalized medicine these modifiers play a crucial role in the disease's prognosis, and future studies to address those factors are clearly warranted.

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FIELD OF VISION

Diagnostic criteria for autoimmune hepatitis in children: A challenge for pediatric hepatologists

Priscila Menezes Ferri, Alexandre Rodrigues Ferreira, Débora Marques Miranda, Ana Cristina Simões e Silva

Priscila Menezes Ferri, Alexandre Rodrigues Ferreira, Division of Pediatric Gastroenterology and Hepatology, Department of Pediatrics, School Medicine, Federal University of Minas Gerais, Minas Gerais 30130-100, Brazil
Débora Marques Miranda, Ana Cristina Simões e Silva, National Institute of Science and Technology of Molecular Medicine, Faculty of Medicine, Federal University of Minas Gerais, Minas Gerais 30130-100, Brazil
Author contributions: Ferri PM collected the materials and wrote the manuscript; Ferreira AR and Miranda DM discussed the topic and revised the text; Simões e Silva AC conceived the manuscript and made the general supervision.
Correspondence to: Ana Cristina Simões e Silva, MD, PhD, National Institute of Science and Technology of Molecular Medicine, Faculty of Medicine, Federal University of Minas Gerais, Avenida Alfredo Balena 190, Belo Horizonte, Minas Gerais 30130-100, Brazil. acssilva@hotmail.com
Telephone: +55-31-93600007 Fax: +55-31-34099772
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Abstract

Autoimmune hepatitis (AIH) is a progressive inflammatory liver disorder that is rare in children and adolescents. AIH has a broad clinical spectrum and a quick response to treatment with corticosteroids and immunosuppressive medication. The available diagnosis criteria have limitations and should be evaluated in pediatric populations. Recently, some studies reported that the 2008 simplified diagnostic criteria for AIH could be used in children with high sensibility and specificity. In addition, the authors reported that globulin and immunoglobulin G levels can be used interchangeably for diagnostic purposes. They also demonstrated that the 2008 simplified criteria fail in identifying patients with fulminant hepatic failure. Here, we discuss the limitations of the use of these criteria in pediatric patients and the requirement of more studies to improve the diagnosis of AIH in children.

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Key words: Child; Autoimmune hepatitis; Liver diseases; Diagnosis; Autoimmunity

Peer reviewer: Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of General Medicine 2 Unit, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University Hospital of Pisa, University of Pisa, Via Roma 67, 56124 Pisa, Italy

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INVITED COMMENTARY ON HOT ARTICLES

Autoimmune hepatitis (AIH) is particularly aggressive in children and progresses rapidly unless immunosuppressive treatment is promptly started. The recent publication by Mileti *et al*[1] on the validation of simplified diagnostic criteria for autoimmune hepatitis in children caught our attention for its updated theme, with great relevance to pediatrics and hepatologists. We highly recommend reading the manuscript.

AIH is a progressive inflammatory liver disorder that is rare in children and adolescents. It affects patients who have lost immune tolerance to liver self-antigens[2-4]. It predominates in females, and it is serologically characterized by high levels of transaminases and immunoglobulin G (IgG) and the presence of auto-antibodies and histologically characterized by interface hepatitis in the absence of a known etiology[3]. The age of onset of AIH ranges from 6 mo to 75 years; however, it is rare

before 2 years, and the incidence is higher between 10 and 30 years[3].

The clinical spectrum is broad; asymptomatic patients may only have laboratory abnormalities. Patients may also present with clinical symptoms similar to acute viral hepatitis and even severe liver failure (acute, chronic or fulminant)[3]. Auto-antibodies, such as antinuclear antibodies (ANA), anti-smooth muscle antibody (SMA) and anti-liver/kidney microsome type 1 (anti-LKM1), are important for the diagnosis of AIH. Based on the pattern detected, AIH can be classified into two types: type 1, in which ANA and/or anti-SMA are detected, and type 2, in which anti-LKM1 is detected[5].

Some histological findings can suggest the presence of AIH: piecemeal necrosis with periportal/periseptal lymphocytic infiltrate; interface hepatitis with the destruction of hepatocytes at the periphery of the lobule and erosion of the limiting plate; hepatic regeneration with "rosette" formation; and "bridging collapse", in which connective tissue collapses and expands from the portal area into the lobule[6].

Treatment with corticosteroids and immunosuppressive agents is usually efficient in controlling AIH[3]. Prednisone alone or combined with azathioprine is the main treatment, and it is aimed at reducing liver inflammation and inducing clinical remission and better survival rates. The treatment response is characterized by clinical improvement and the reduction of aminotransferase levels to normal or up two times the highest reference value[3].

Given the necessity to standardize the diagnosis and establish early treatment, in 1993, the International Autoimmune Hepatitis Group (IAIHG) created diagnostic criteria for AIH. Those criteria were revised in 1999 to improve specificity and simplify the scoring system, as cited in the discussion section of the manuscript[1,7]. Simplified diagnostic criteria were established in 2008 to make their use in clinical practice easier.

Mileti *et al*[1] selected 37 children under 21 years old with a diagnosis of AIH and 40 children diagnosed with other liver diseases and evaluated the sensitivity and specificity of the diagnostic criteria proposed in 2008. They found a sensitivity of 87% and a specificity of 89% for the 2008 criteria and also observed that these criteria did not rank patients with signs of fulminant hepatic failure (FHF) well. The authors also compared the use of IgG and serum globulin levels to grade the criteria and concluded that they may be used interchangeably without impairing the final score.

The use of 2008 diagnostic criteria might simplify the day-to-day work of hepatologists and might provide an interesting measure for pediatric patients. Based on the cited results and considering that autoimmune hepatitis is important in the differential diagnosis of liver disease in childhood, we discuss some important points that may affect the use of these criteria.

Use of diagnostic criteria in pediatric patients

In general, the adoption of diagnostic criteria is considered to be an attempt to standardize observations and clinical procedures for different centers with the main goal of a "common language". Diagnostic criteria should contain well-defined measures and should be easy to apply in clinical practice to facilitate disease classification, diagnosis and, consequently, treatment. Another important issue concerning diagnostic criteria is the possibility of distinguishing patients who need to be assigned to different therapies or management strategies, even if the diagnosis is not clearly established. This important feature of the diagnostic criteria will certainly allow early treatment, which might improve the outcome of patients.

For many diseases and clinical conditions, we already have diagnostic criteria that were usually established for the adult population and subsequently adapted to pediatric patients. The ideal situation would be that the diagnostic criteria used for children arise from studies on this age group.

Another important point is that diagnostic criteria raise the possibility of a disease, but in most cases, it is difficult to exclude other diagnoses. For example, the diagnosis of rheumatic fever with the criteria originally proposed by Jones[8] in 1944 includes a combination of arthritis, fever, and a high sedimentation rate in the presence of a recent Group A streptococcal infection. However, many children with juvenile arthritis also present with exactly the same features[9].

For AIH, the same problem occurs, with the criteria being initially established for adults and later adopted with changes for pediatric patients. In its first version in 1993, based on consensus from the IAIHG, the diagnosis of AIH in the pediatric population was not considered to require separate diagnostic criteria, as cited by Mileti *et al*[1]. Two other studies attempted to evaluate recent criteria (1999 and the simplified criteria created in 2008) in pediatric populations, and they had controversial results. Those studies are listed and compared with the Mileti *et al*[1] study in Table 1.

The authors generally concluded that the 1999 and 2008 diagnostic criteria could be used in children with good sensitivity and specificity in most cases, but they also showed some limitations. First, in children with the final diagnosis of primary sclerosing cholangitis (PSC), the study of Ebbeson *et al*[10] found that the 1999 criteria adequately scored these patients as "not AIH", while Hiejima *et al*[11] showed that using both criteria, all 5 children with PSC were graded as having AIH. Considering these findings and the possibility of AIH/PSC overlap syndrome in children, the use of gamma-glutamyl transferase could improve the specificity of the 1999 criteria but would not improve the 2008 criteria. Therefore, the recent recommendation of performing cholangiographic evaluation in all children with an initial diagnosis of AIH was added[12,13].

The second issue is the limitation cited by Mileti *et al*[1] regarding the reliability of the 2008 criteria in identifying patients with FHF. The acuteness and severity of FHF

Table 1 Summary of pediatric studies on the validation of diagnostic criteria for autoimmune hepatitis

Authors	Place and date	*n*	Diagnostics	Tested criteria	Results	Conclusions
Mileti *et al*[1]	United States, 2012	68	37 AIH for 1999 criteria/ 31 AIH for 2008 criteria 40 non-AIH	1999 and 2008	1999 criteria: 29 of 31 subjects (94%) as definite AIH; 2 of 31 subjects (6%) as probable AIH Simplified criteria: 25 of 31 subjects (81%) as definite AIH; 2 of 31 subjects (6%) as probable AIH The 2008 diagnostic criteria had a sensitivity of 87% and a specificity of 89% and did not identify 4 patients with AIH and fulminant hepatic failure	The 2008 criteria showed high levels of sensibility and specificity Patients with fulminant hepatic failure need the 1999 criteria Globulin and immunoglobulin G can be used interchangeably
Ebbeson *et al*[10]	Canada, 2004	28	21 AIH 4 PSC 3 ASC	1999	18 of 21 (86%) with AIH scored as definite AIH and 3 of 21 (14%) scored as probable All patients with isolated PSC scored as not AIH	The IAIHG scoring system has a use in children Using the GGT ratio may improve the specificity for children
Hiejima *et al*[11]	Japan, 2011	56	20 AIH 36 non-AIH liver diseases	1999 and 2008	Sensitivity and specificity of the 1999 criteria were 100% and 81%, respectively Sensitivity and specificity of the simplified criteria were 55% and 86%, respectively All 5 children with PSC were graded as having AIH by both criteria	The specificity of the simplified AIH criteria is high Simplified criteria could not differentiate between AIH and PSC and do not seem to be a reliable diagnostic tool in children

AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; ASC: Autoimmune sclerosing cholangitis; IAIHG: International autoimmune hepatitis group; GGT: Gamma-glutamyl transferase.

demand a rapid and precise diagnostic definition. If the diagnostic criteria provide information in this special situation, treatment with corticosteroids could be started promptly. Thus, further research is important to identify factors or measurements that could improve the diagnosis of FHF.

The third issue is that the auto-antibodies used for diagnosis often have lower titers in children than the cut-off values considered positive in adults and utilized in both diagnostic criteria (1999 and 2008)[4]. The reactivity of ANA, SMA and anti-LKM1 is low; therefore, titers of 1/20 for ANA and SMA and 1/10 for anti-LKM1 can be considered relevant in children[4]. The 2008 and 1999 criteria consider titers above 1/40 to be significant, and the laboratory test is performed with a minimum dilution of 1/40; therefore, children with titers of 1/20 could be false negative for the tested auto-antibody. Therefore, in children with clinical history and a physical examination compatible with AIH, we should not exclude this diagnosis when negative auto-antibody results are obtained.

The fourth issue is the use of histological findings as one point of the diagnostic criteria for AIH. Histology was included in the 1993 and 1999 criteria, and each feature of the liver biopsy was scored as a separate item[7]. In contrast, the simplified criteria utilize only two parameters: histology compatible with AIH and histology typical of AIH[14]. The concern is that a biopsy is not possible at the beginning of follow-up in many pediatric cases because these patients usually exhibit significant liver dysfunction and coagulopathy. Thus, sometimes, clinicians do not have this information and face the necessity of initiating corticosteroid therapy without knowledge of the histological condition. Björnsson *et al*[15] questioned the importance of histology in the diagnosis of typical cases of AIH and concluded that the majority of patients with typical laboratory features of AIH are likely to have compatible liver histology. Not surprisingly, liver biopsy may reveal another hepatic disease that might affect clinical management. We agree that whenever possible, liver biopsy should be performed prior to initiating immunosuppressive therapy in patients with AIH. However, we also believe that if it is not possible to obtain initial histological findings, the initiation of treatment for highly suspected patients should not be delayed. Indeed, for the majority of cases with suggestive clinical features and compatible laboratorial data, the diagnosis of AIH can be reliably established in the absence of liver histology. Ultimately, liver biopsy should be performed, whenever possible, to differentiate other hepatic diseases and according to the guidelines of the American Association for the Study of Liver Disease Practice Guidelines for Autoimmune Hepatitis[13].

The last point to be addressed is the sample size of the Mileti *et al*[1] cross-sectional study, which was composed of just a few individuals classified with both criteria. Similar to other pediatric studies on AIH, the sample size is small. Additional studies should be performed with other populations to establish the best diagnostic criteria for pediatric AIH and to address all other points that require further elucidation in AIH and hepatic-related disorders.

In conclusion, the use of 2008 diagnostic criteria is an important tool in the diagnosis of AIH in children, and the study of Mileti *et al*[1] clearly corroborates this view. However, considering the existing criteria, more studies with larger series seem to be necessary to validate their use in pediatric patients. Finally, we believe that additional pediatric studies on AIH might allow for the clear differentiation between AIH and PSC, include

alternative ways to define fulminant hepatic failure and establish lower auto-antibody titers for pediatric patients.

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FIELD OF VISION

Asymptomatic pancreatic lesions: New insights and clinical implications

Martin Loos, Christoph W Michalski, Jörg Kleeff

Martin Loos, Christoph W Michalski, Jörg Kleeff, Department of Surgery, Klinikum rechts der Isar, Technische Universität München, 81675 München, Germany
Author contributions: All authors collected the material; Loos M drafted the article; Michalski CW and Kleeff J critically revised the article for important intellectual content; and all authors approved the final version of the manuscript.
Correspondence to: Jörg Kleeff, MD, AGAF, FACS, Department of Surgery, Klinikum rechts der Isar, Technische Universität München, Ismaninger Strasse 22, 81675 München, Germany. kleeff@tum.de
Telephone: +49-89-41405098 Fax: +49-89-41404870
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Abstract

Despite great efforts in experimental and clinical research, the prognosis of pancreatic cancer (PC) has not changed significantly for decades. Detection of pre-invasive lesions or early-stage PC with small resectable cancers in asymptomatic individuals remains one of the most promising approaches to substantially improve the overall outcome of PC. Therefore, screening programs have been proposed to identify curable lesions especially in individuals with a familial or genetic predisposition for PC. In this regard, Canto *et al* recently contributed an important article comparing computed tomography, magnetic resonance imaging, and endoscopic ultrasound for the screening of 216 asymptomatic high-risk individuals (HRI). Pancreatic lesions were detected in 92 of 216 asymptomatic HRI (42.6%). The high diagnostic yield in this study raises several questions that need to be answered of which two will be discussed in detail in this commentary: First: which imaging test should be performed? Second and most importantly: what are we doing with incidentally detected pancreatic lesions? Which ones can be observed and which ones need to be resected?

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Key words: Pancreatic cancer; Early-stage pancreatic cancer; Asymptomatic high-risk individuals; Pre-invasive pancreatic lesions; Cystic pancreatic tumors; Screening; Computed tomography; Magnetic resonance imaging; Endoscopic ultrasound

Peer reviewers: Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University of Pisa, Director of General Medicine 2 Unit University Hospital of Pisa, Via Roma 67, 56124 Pisa, Italy; Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, CA, 5901 E. Seventh Str., Long Beach, CA 90822, United States; De Aretxabala Xabier, Professor of Surgery, University of Chile, Santos Dumont 999, 8380000 Santiago, Chile

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INVITED COMMENTARY ON HOT ARTICLES

With great interest we noticed the article published by Canto *et al*[1], which investigated the prevalence and characteristics of pancreatic lesions in high-risk individuals (HRI) for pancreatic cancer (PC).

Up to 10% of PC cases are attributed to a familial or inherited predisposition that can substantially increase the risk for PC[2-7]. For example, patients suffering from Peutz-Jeghers syndrome have a 132-fold increased risk for PC[8]. Other genetic predispositions include hereditary pancreatitis (*PRSS1* gene mutations, lifetime risk for PC of up to 40%)[9,10], the familial atypical multiple

mole melanoma syndrome (*p16/CDKN2A* gene mutations, 13-fold to 22-fold increased risk)[11,12], the Lynch syndrome (mismatch repair gene mutations, 8.6-fold increased risk)[13,14], familial adenomatous polyposis (*APC* gene mutations, 4.5-fold increased risk)[15,16], the familial breast-ovarian cancer syndrome (*BRCA1/2* gene mutations, 2.3-fold to 10-fold increased risk)[6,7,17,18], and individuals with a strong history of PC (at least two first-degree relatives with PC, 6.4-fold to 32-fold increased risk)[2,19]. For these, HRI screening programs have been proposed to detect early-stage pancreatic cancers or even pre-invasive lesions, which are potentially curable because once PC progresses into advanced stages the chance for cure decreases abruptly. In the study by Canto *et al*[1], pancreatic lesions were detected in 92 of 216 HRI (42.6%). The confirmed or suspected final diagnosis included branch-duct intraductal papillary mucinous neoplasm (IPMN) (n = 82), combined-duct IPMN (n = 2), main-duct IPMN (n = 4), and pancreatic endocrine tumor (n = 3)[1]. Such a high prevalence of pancreatic lesions is rare but has been reported before by Verna *et al*[20] who detected pancreatic lesions in 11 of 33 HRI (33.3%) and 14 of 31 HRI (45%) by magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS), respectively. A possible explanation for the high diagnostic yield is the high quality of the screening tests and the highly experienced team of radiologists and gastroenterologists involved in the diagnostic work-up in both studies. On the other hand, it is well known that the prevalence of pancreatic lesions increases with age. Therefore, the age at which the screening test was performed is crucial. In the study by Canto *et al*[1], the mean age of HRI at screening was 56.1 years which is comparable to other published studies. When compared to individuals in the general population, the baseline diagnostic yield was significantly higher in HRI. Incidental cystic pancreatic lesions can be found in up to 2.8% in the general population[21] and this number will increase with better imaging tests. These findings point towards the importance of screening initiatives for HRI with high-resolution imaging tests but also illustrate the dilemma we are currently facing. The more diagnostic imaging studies we perform, the more lesions we will find - even in asymptomatic individuals without increased PC risk. The question that remains is what to do with these incidental lesions. How accurate is our interpretation of an incidental pancreatic lesion? Which lesions really represent a precursor of PC and will proceed to invasive cancer? How many of these lesions already carry incipient cancer? In 2006, the Sendai consensus proposed guidelines for the management of cystic lesions including IPMN and mucinous cystic neoplasms (MCN), which have been widely adopted[22]. However, the diagnosis and subsequent recommendation for pancreatic resection are based on imaging in combination with fine needle aspiration from cystic lesions and confirmation of the presumed diagnosis can only be made after surgical resection. A recent study published by Correa-Gallego *et al*[23] reported a series of 330 patients with incidentally discovered cystic neoplasms of the pancreas from a high volume center for diseases of the pancreas. One-hundred-thirty-six patients (41%) were operated on at diagnosis. Preoperative and final histological diagnoses were correlated[23]. The accuracy of preoperative diagnoses was only 64% for presumed branch-duct IPMN (32 of 50 cases)[23]. Ten of the 18 patients (20%) had an extension to the main duct leading to the final diagnosis of combined-duct IPMN[23]. The diagnostic accuracy for presumed MCN was also only 60% (18 of 30 cases)[23]. Of all patients who were operated on, 6 had an invasive carcinoma (2 branch-duct IPMN, 3 main-duct IPMN, and 1 MCN) and 19 patients had a carcinoma in situ (8 main-duct IPMN, 8 cystic pancreatic endocrine neoplasms, and 3 others)[23]. Therefore, correct interpretation of pancreatic lesions is still problematic and even with established guidelines choosing the adequate treatment remains challenging because the final diagnosis can only be verified after surgical resection.

Another problem is that IPMN are usually multifocal which has been addressed by the concept of the field defect of pancreatic duct instability[24-27]. Pancreata which harbour an IPMN are at increased risk of developing carcinoma. Several studies of patients with IPMN reported synchronous or metachronous invasive PC and these cancers were also present in areas distant from the index IPMN[28-30]. The most recent study by LaFemina *et al*[31] analyzed the prevalence and site of PC progression in 157 patients with suspected or confirmed IPMN who were initially selected for radiographic surveillance. After a median length of surveillance of 15 mo (range: 6-193 mo), 97 patients (62%) eventually underwent resection[31]. Surgical pathology confirmed 18 cases of invasive carcinoma (11%), which were diagnosed at a median of 24 mo after the initial diagnosis of IPMN[31]. Ten patients had main-duct IPMN (56%), 5 had branch-duct (28%), and 3 had combined-duct (17%) IPMN[31]. Four patients (22%) developed PC in a region of the pancreas distinct from the radiographically identified IPMN[31]. Miller *et al*[32] followed 153 patients after pancreatic resection for IPMN with clear resection margins. The authors found that 31 patients developed de novo IPMN in the pancreatic remnant and in 3 cases an invasive carcinoma was diagnosed[32]. Therefore, the confirmation of IPMN requires continuous surveillance of the entire pancreatic gland or of the pancreatic remnant after previous resection[32].

In this regard, Canto *et al*[1] attempted to answer the question of which imaging test should be performed for screening HRI and compared computed tomography (CT), MRI and EUS for detecting pancreatic lesions. The authors found that EUS and MRI detected pancreatic lesions better than CT[1]. The baseline diagnostic yield for EUS, MRI, and CT was 42.6%, 33.3% and 11%, respectively[1]. The authors' conclusion that EUS and MRI are currently the best initial tests for detecting early pancreatic lesions is supported by other studies[33-37]. Main limitations of CT include not only its poor sensitivity for small pancreatic lesions (< 10 mm) which

is important for screening for early pancreatic neoplasms but also the use of ionizing radiation which has recently raised concerns regarding the increased risk of radiation-related cancers associated with CT[38]. However, multi-detector CT remains the most widely used imaging modality because of its high accuracy for detecting solid tumors and staging of pancreatic malignancies, its cost effectiveness and its non-invasive nature. Furthermore, EUS and MRI are more cost-intensive and both tests are more dependent on the experience of the performing and diagnosing gastroenterologist and radiologist. Although the invasive nature of EUS as an endoscopic procedure further limits its role in a screening program, the possibility of EUS-guided fine-needle aspiration of pancreatic cystic lesions may be of diagnostic value especially when malignant cells can be detected. Future work with molecular analysis of cyst fluid, direct cystoscopy, and confocal laser endomicroscopy may further enhance its diagnostic accuracy[39].

Based on the high diagnostic yield of modern high-resolution imaging tests, it appears to be reasonable to routinely screen HRI. Based on current evidence, MRI (and EUS) should be the initial imaging tests to be performed. The question at what age screening of HRI should start has yet to be answered.

Although we have learned a lot in the past two decades about the nature especially of pancreatic cystic lesions, we are still facing a great challenge of how to manage incidental pancreatic lesions. Canto *et al*[1] suggest that the goal of PC screening and surveillance programs should be to detect and selectively treat asymptomatic high-grade precursor neoplasms rather than focussing on detection of invasive cancers. However, especially because IPMN constitute a heterogeneous group of pancreatic cystic neoplasms, a better understanding of the natural history of IPMN and its subtypes is necessary to distinguish lesions that need immediate surgical resection and those that can be safely observed. Not only a better understanding of patient characteristics and further progress in imaging tests are needed but also the identification of reliable biomarkers that can be used to identify pancreatic lesions that are about to proceed to PC in asymptomatic individuals.

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FIELD OF VISION

Opposite fates of fructose in the development of metabolic syndrome

Marta Alegret, Juan C Laguna

Marta Alegret, Juan C Laguna, Department of Pharmacology and Therapeutic Chemistry, School of Pharmacy, University of Barcelona, Institute of Biomedicine, 08028 Barcelona, Spain
Marta Alegret, Juan C Laguna, Biomedical Network Research Centre, Physiopathology of Obesity and Nutrition (CIBERobn), 08028 Barcelona, Spain
Author contributions: Alegret M and Laguna JC contributed equally to the writing of this manuscript.
Correspondence to: Dr. Juan C Laguna, Department of Pharmacology and Therapeutic Chemistry, School of Pharmacy, University of Barcelona, Institute of Biomedicine, Avda. Diagonal 643, 08028 Barcelona, Spain. jclagunae@ub.edu
Telephone: +34-93-4024530 Fax: +34-93-4035982
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Abstract

This short review comments on the recently published work of Ishimoto *et al* regarding the opposing effects of fructokinase C and A isoforms on fructose-induced metabolic syndrome in mice. The framework for the commentary is the preexisting background of epidemiological and experimental data regarding the association between ingestion of fructose, as present in sweetened beverages, and the development of metabolic syndrome. The work of Ishimoto *et al* clearly confirms the negative effect of fructose on lipid and glucose metabolism, independently from the amount of energy provided by the ingested sugar. It also confirms the absolute requirement of liver fructose metabolism, driven by fructokinase activity, in order to develop the full spectrum of metabolic syndrome alterations.

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Key words: Lipid metabolism; Fatty liver; Obesity; Hyperglycemia; Dyslipidemia

Peer reviewers: Astrid van der Velde, PhD, AMC Liver Center, Meibergdreef 69-71, 1105 BK Amsterdam, The Netherlands; Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, CA, 5901 E. Seventh Str., Long Beach, CA 90822, United States; Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of General Medicine 2 Unit, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University Hospital of Pisa, University of Pisa, Via Roma 67, 56124 Pisa, Italy

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INVITED COMMENTARY ON HOT ARTICLES

Non-communicable diseases continue to place an enormous burden on humanity, and have recently surpassed infectious diseases as the primary concern for human health. Among the former, cardiovascular events derived from the development of atherosclerosis associated with chronic metabolic diseases (such as obesity, type 2 diabetes mellitus and metabolic syndrome) are increasing all over the world. Besides genetic predisposition, two lifestyles options that are deeply rooted in affluent Western societies are fuelling this vicious trend: a lack of physical activity and the consumption of hypercaloric diets which tip people's energy balance to an overwhelming excess of ingested, unused energy. Dietary changes favor the consumption of processed, palatable, high-calorie-density foodstuffs over traditional ones. Among the former are the whole spectrum of sweetened beverages (fruit juices, sodas, milk shakes, *etc.*) which are sweetened with High Fructose Corn Syrup or sucrose. In both cases, such beverages offer the ingestion of large amounts of two simple sugars, glucose and fructose, in similar proportions[1,2].

When ingesting calories in liquid form, as in the case of the consumption of sugar-sweetened beverages, there is a lack of an appropriate compensatory response and thus no corresponding adequate reduction in the ingestion of calories provided by solid food. Therefore, it favors an excessive daily intake of calories. However, besides the increase in ingested calories, the particularities of fructose metabolism also need to be considered. Fructose is the carbohydrate with the greatest ability to induce hypertriglyceridemia, in part due to a more marked increase in hepatic lipogenesis than that resulting from a similar intake of glucose[3,4].

After entering liver cells, fructose is rapidly metabolized by the enzyme fructokinase to fructose-1-phosphate. Fructose feeding induces its own metabolism by increasing fructokinase expression. The product of fructokinase (fructose 1-phosphate) is further metabolized by the enzyme aldolase to the triose phosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The former enters directly into the glycolytic pathway producing pyruvate, which is converted to acetyl-CoA and citrate inside the mitochondria, providing the carbon moiety for de novo synthesis of long-chain fatty acids. Meanwhile, dihydroxyacetone phosphate is converted into glycerol 3-phosphate, which provides the glycerol backbone of triglycerides. Since hepatic fructose metabolism bypasses the main rate-controlling enzyme of glycolysis, phosphofructokinase, a high flux of this carbohydrate to the liver results in a marked increase in lipogenesis and in the production of very low density lipoproteins. In contrast, glucose is first phosphorylated in position 6 by glucokinase, followed by isomerization to fructose-6-phosphate, which is converted by phosphofructokinase into fructose 1, 6-diphosphate before entering the glycolytic pathway. The progression of glucose to complete glycolysis is under the control of phosphofructokinase activity, which is very tightly regulated by the products of the glycolytic pathway, citrate and ATP. Thus, when large amounts of glucose are consumed, there is a feedback inhibition of glycolysis and glucose uptake, limiting pyruvate production, and the lipogenic effect is not as intense as in the case of fructose[5]. Furthermore, research from our laboratory has shown that fructose, but not glucose at a similar energy intake, can down-regulate the liver peroxisome proliferator activated receptor (PPAR) system, impairing fatty acid oxidation, and thus helping the accumulation of fatty acids for triglyceride accretion[6]. At least in male rats, the effect of fructose on the PPARα system is related to the induction of a clear state of liver leptin-resistance[7].

Fructokinase, and its capacity to respond to fructose increasing its expression[8-10] thereby facilitating the incorporation of fructose into liver metabolism, is of paramount importance in the manifestation of metabolic disturbances induced by fructose ingestion. For example, Ouyang *et al*[11] have shown that patients with non alcoholic fatty liver disease consume nearly approximately 2-fold to 3-fold more fructose than controls, primarily in the form of sweetened beverages, and present higher liver expression of fructokinase. In an experimental model of male rats supplemented with 10 % w/v fructose solution, we found that atorvastatin, a hypocholesterolemic drug, counteracts fatty liver and inflammation by reducing the fructose-related liver fructokinase induction[12].

All this background information is capital to clearly appreciate the real importance of the information provided by Ishimoto *et al*[13] in their paper published recently in Proceedings of the National Academy of Sciences. Fructokinase exists in two isoforms, fructokinase A and fructokinase C, with differentiated patterns of tissue expression. Fructokinase C is primarily expressed in liver, kidney and intestines, while the A isoform is expressed ubiquitously[14]. Due to the lower K_m of fructokinase C, this isoform is held to be mainly responsible for fructose metabolism[15]. By cleverly using the knock-out mice that developed themselves for both isoforms (KO-A/C) or only for isoform A (KO-A) and two patterns of fructose supplementation (15% and 30% w/v solutions of fructose in drinking water for 25 wk), Ishimoto *et al*[13] clearly delineated the roles of fructokinase A and fructokinase C in the induction of the manifestations of metabolic syndrome by fructose.

While wild-type mice supplemented with 15% fructose presented almost the full panoply of metabolic syndrome manifestations (obesity, hyperglucemia, hyperinsulinemia, hyperleptinemia and fatty liver), KO-A/C mice supplemented with 30% fructose, despite ingesting equivalent amounts of fructose, presented no manifestations of metabolic syndrome at all. In contrast, KO-A mice supplemented with 30% fructose, although ingesting the same amount of fructose as their matching wild-type controls, presented a more severe manifestation of metabolic syndrome than their wild-type counterparts. Ishimoto *et al*[13] carefully confirmed, by using recombinant fructokinase A, that this isoform, although having a low affinity for fructose, is capable of metabolizing fructose at a range of physiological concentrations.

In our opinion, three clear facts can be derived from the results presented by Ishimoto *et al*[13]: (1) KO-A/C mice were protected from developing metabolic syndrome, despite having a similar fructose intake and energy balance as their wild-type counterparts, which did indeed, develop metabolic syndrome. This experiment clearly demonstrates that the induction of metabolic syndrome by fructose does not depend on the amount of energy ingested (similar in KO-AC and wild-type control mice), instead it depends on the metabolism of ingested fructose, which clearly depends on the presence of fructokinase activity in tissues; (2) The fructokinase A isoform plays a physiological role in the metabolic fate of fructose. Its absence increases fructose concentration in plasma, even in animals ingesting only tap water; and (3) Furthermore, the absence of fructokinase A activity, as in KO-A mice, which shifts the whole metabolism of fructose to the C isoform, worsens the metabolic alterations induced by fructose ingestion. As mentioned above, fruc-

tokinase C is mainly expressed in liver and kidney and, to a lesser extend, intestines. If we consider that, after fructose ingestion, the sugar is rapidly and almost completely extracted by the liver through the glucose transporter 2[16], these results clearly point to the liver as the main target organ of fructose, and the one whose metabolic alteration finally results in the manifestation of metabolic syndrome in the whole organism. It would be very interesting to generate KO-C mice and supplement them with fructose in order to definitely confirm this claim.

While recognizing the problems of directly transposing experimental results obtained from animal models to the everyday-life of human consumption of sweetened beverages, the work of Ishimoto *et al*[13] demonstrates that fructose especial metabolism clearly matters as a key factor in the development of pathologies associated with the metabolic syndrome.

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FIELD OF VISION

Entry of hepatitis C virus into the cell: A therapeutic target

José Antonio Del Campo, Ángela Rojas, Manuel Romero-Gómez

José Antonio Del Campo, Ángela Rojas, Manuel Romero-Gómez, Unit for Medical and Surgical Management of Digestive Diseases, Center for Biomedical Research in Digestive and Liver Diseases Network (CIBERehd), Valme University Hospital, University of Seville, E-41014 Sevilla, Spain
Author contributions: Del Campo JA, Rojas A and Romero-Gómez M designed the study, performed bibliographic search and wrote manuscript and revision.
Correspondence to: Manuel Romero-Gómez, Professor, Unit for Medical and Surgical Management of Digestive Diseases, Center for Biomedical Research in Digestive and Liver Diseases Network (CIBERehd), Valme University Hospital, University of Seville, Avda. Bellavista s/n, E-41014 Sevilla, Spain. mromerogomez@us.es
Telephone: +34-95-5015761 Fax: +34-95-5015899
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Abstract

Several receptors have been identified as implicated on viral entry into the hepatocyte; and, this interaction between the virus and potential receptors could modulate infection, spontaneous viral clearance, persistence of the infection and the widespread of the virus as outbreak. Nevertheless, the playing role of each of them remains controversial. The Niemann-Pick type C1 like 1 gene (*NPC1L1*) receptor has been recently implicated on hepatitis C virus (HCV) entry into the cell and ezetimibe, an anti-cholesterol drug seems to block that, emerging the idea to control hepatitis C outbreak modulating lipid-related receptors. Hepatitis C infection seems to modulate lipid metabolism according to host genetic background. Indeed, it circulates like a lipoviroparticle. The main aim of this field of vision would be to discuss the role of hepatocyte receptors implicated on virus entry, especially NPC1L1 and the therapeutic options derived from the better knowledge about HCV-lipids- receptors interaction.

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Key words: Hepatitis C virus entry; Niemann-Pick type C1 like 1 gene; Lipid metabolism; Ezetimibe

Peer reviewer: Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of General Medicine 2 Unit, Di-rector of Liver and Digestive Disease Division, Department of Internal Medicine, University Hospital of Pisa, University of Pisa, Via Roma 67, 56124 Pisa, Italy

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INVITED COMMENTARY ON HOT ARTICLES

Viral entry is the first step of virus-host cell interactions and, is a highly orchestrated process involving several viral and host cell factors. Indeed, the virus is able to escape from neutralizing antibodies and promote direct cell-cell transmission. Understanding the mechanisms of viral entry and escape is a prerequisite to define the viral and cellular targets that will give broad protection against hepatitis C virus (HCV) infection. HCV is an enveloped single-strand RNA virus that mainly targets hepatocytes. Due to the difficulty to grow HCV *in vitro* and the species specificity of this virus, surrogate model systems have been developed to study HCV entry into hepatocytes: recombinant envelope glycoproteins[1], HCV-like particles[2], HCV pseudo-particles[3,4] and recombinant infectious HCV[5-7] have been used to study the interactions of the viral envelope with human hepatoma cells or primary human hepatocytes. Four cellular factors have been described as essential for HCV entry (Figure 1): the tetraspanin molecule CD81, the scavenger receptor class B member I and the tight junction proteins claudin-1 and occludin[8]. Interestingly, neutralizing antibodies against CD81 are able to block HCV entry *in vitro* and also in im-

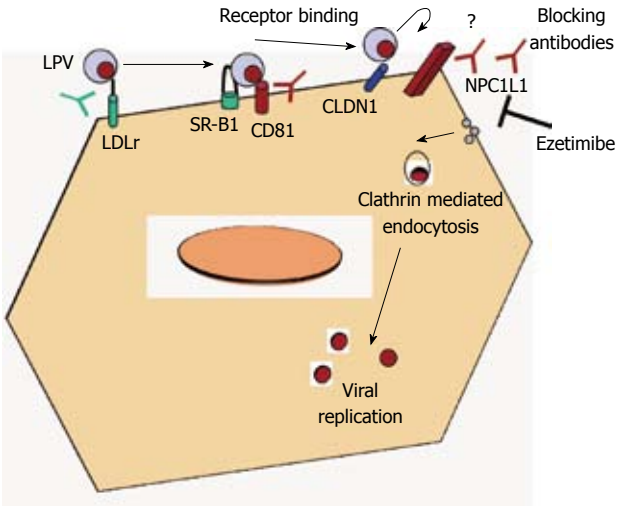


Figure 1 Schematic representation of viral receptors in hepatocytes. Possible mechanisms of viral entry blockade are also depicted by using specific antibodies or inhibitory drugs. LDLr: Low-density lipoprotein receptor; NPC1L1: Niemann-Pick C1-like 1 cholesterol absorption receptor; SR-B1: Scavenger receptor class B type 1; LPV: Lipoviroparticle; CLDN1: Claudin-1.

munodeficient mice transplanted with human hepatocytes, the best currently available small animal model of HCV infection[9]. Neutralizing antibodies against the HCV envelope proteins E1 and E2 could also represent a promising approach to avoid HCV infection, but the main challenge here is the enormous genetic variability of the virus. The requirement for sequential interactions between the viral envelope and key host receptors/co-receptors may provide new drug targets that could be exploited by small-molecule inhibitors. Recently, Syder *et al*[10] discovered and optimized a series of 1,3,5-triazine compounds that are potent, selective and non-cytotoxic inhibitors of HCV entry. Representative compounds fully suppress both cell-free virus and cell-to-cell spread of HCV *in vitro*. To date, only one oral HCV entry inhibitor with a defined mechanism of action, ITX-5061, has entered clinical testing (phase 2a)[11]. ITX-5061 binds directly to SR-B1 and blocks a key post-binding step in the viral entry process. In chronically infected patients we cannot find a single isolate of HCV but rather a population of related yet different viral variants (quasispecies), containing a vast repertoire of preformed variants that allow rapid escape from selective pressures such as neutralizing antibodies or anti-viral drugs. Recently, two well-known molecules have been shown to inhibit HCV entry: the green tea catechin epigallocatechin-3-gallate (EGCG) and the tyrosine kinase inhibitor erlotinib. Erlotinib blocks HCV entry by inhibition of the activity of the epidermal grow factor -receptor which is required for formation of CD81-claudin-1 co-receptor associations[12]. EGCG inhibits viral attachment to the target cell as well as cell-to-cell transmission between adjacent cells.

Role of Niemann-Pick C1-like 1 cholesterol absorption receptor in viral entry

Sainz *et al*[13] have recently discovered a novel surface receptor involved in HCV entry, the Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1), a 13transmembrane-domain cell surface cholesterol-sensing receptor, expressed on the apical surface of intestinal enterocytes and human hepatocytes, including Huh7 cells, is responsible for cellular cholesterol absorption and whole-body cholesterol homeostasis. NPC1L1 was first identified as a homolog of Niemann-Pick C1 protein[14], the deficiency of which causes Niemann-Pick disease type C1, a genetic disorder characterized by intracellular accumulation of unesterified cholesterol in the endosomal/lysosomal system of neurons that causes neurodegeneration and premature death[15,16]. Human *NPC1L1* gene maps to chromosome 7p13, spans 29 kb, encodes a 5 kb mRNA and predominantly produces a protein of 1332 amino acids[17]. NPC1L1 locates to the brush border membrane of the enterocyte and the canalicular membrane of the hepatocyte. Biliary cholesterol, which is secreted into bile by hepatocytes, accounts for more than two thirds of the total amount of cholesterol in the gut lumen. Since the cholesterol is water-insoluble, it is delivered to the brush border membranes by bile salt micelles.

These authors have shown that using NPC1L1-specific antibody, HCV infection was reduced in a similar way that CD-81 specific antibodies did. Ezetimibe is a 2 azetidinone-class drug that has been approved by the Food and Drug Administration as a cholesterol-lowering medication[18]. Sainz *et al*[13] also shown the role of NPC1L1 receptor on HCV infection *in vivo*, using immunodeficiency mice repopulated with human hepatocytes and treated them *via* oral gavage with ezetimibe. They have demonstrated that ezetimibe treatment delayed the establishment of HCV infection in mice pretreated for 2 wk, confirming the ability of this drug to inhibit HCV infection *in vivo*. However, ezetimibe concentration in those experiments was high (30 μmol/L). That means for a 70 kg weight adult to ingest 84 ezetimibe 10 mg tablets per day, when the usual doses for ezetimibe is one 10 mg tablet per day. So far, an ezetimibe based therapy for HCV does not seem suitable. Probably the use of antibodies against NPC1L1 would be a better alternative.

Lipid metabolism and HCV infection

HCV infection is tightly associated with alterations in lipid metabolism and lipids have been shown to play important roles during the viral replication cycle[19,20]. Indeed, recent studies based on transcriptome and proteomic analyses have demonstrated that expression of host genes involved in the biosynthesis, degradation and transport of intracellular lipids is profoundly altered upon infection. The expression of sterol regulatory element binding proteins, which control transcription of genes required for cholesterol biosynthesis, is stimulated by HCV infection. In agreement with this, the expression of fatty acid synthase (FASN) and other genes related to the synthesis and transport of fatty acids is upregulated in infected cells[21,22]. Moreover, the inhibition of FASN

activity blocks HCV RNA replication and production of infectious virus particles[23]. Finally, expression of genes regulating geranylgeranylation of cellular proteins important for HCV replication is also upregulated in HCV infected cells[21] .

HCV from infected patients (sera) can be precipitated with antibodies against lipoproteins (LP), indicating that HCV circulates associated to LP. Removal of LP by apheresis reduced HCV RNA level by 77%, suggesting that most of the viral particles are tightly associated with lipoproteins[24]. Two different studies[25] suggest that infectious HCV particles are highly associated with LP. Lipoproteins are easily endocytosed, supporting the hypothesis that HCV can use this association to LP to adhere the cell and subsequently enter into the host cell by endocytosis rather than direct fusion to the membrane[26].

Lipids droplets are necessary for the lipoviroparticle formation. HCV is hypothesized to initiate assembly in close association with lipid droplets by coating lipid droplets with the core protein and bringing together nonstructural (NS) and structural proteins in a NS2-dependent manner[27-29]. Following capsid assembly, nascent virions bud into the lumen of the endoplasmic reticulum (ER) where the glycoproteins E1/E2 reside in addition to the very low density lipoprotein (VLDL) secretion machinery. HCV is infectious upon envelopment at the ER, and it is thought that apolipoprotein E (ApoE) is acquired early during assembly because knockdown of ApoE reduces intracellular and extracellular virus; also NS5A interacts with ApoE[30,31]. Core proteins disturb microsomal triglyceride transfer protein (MTP) activity in the hepatocyte[32] and it has been described that NS5A could be interfered with MTP function. MTP is an essential chaperone for the assembly of VLDL, which transfers triglyceride, phospholipids, and cholesterol from the hepatocytes. Reduced activity of MTP results in decreased secretion of VLDL, leading to lipid accumulation. This fact could explain development of steatosis.

The low-density lipoprotein receptor (LDLr) was proposed as a potential entry factor for HCV[33], however, its implication in virus entry remains unclear. Moreover, by using HCV particles isolated from patients, a correlation has been shown between the accumulation of HCV RNA into primary hepatocytes, expression of LDLr messenger RNA, and LDL entry[34]. The potential involvement of the LDLr in HCV entry has also been reported in the HCVcc system[35]. Albecka *et al*[36] have shown that HCV particles can interact with the LDLr. However, this interaction does not necessarily lead to a productive infection. Furthermore, those data indicate a role for the LDLr as a lipid-providing receptor, which modulates viral RNA replication.

Quercetin, an abundant flavonoid found in fruits and vegetable, has been implicated in lowering the risk of cardiovascular disease that is often associated with high plasma levels of LDL cholesterol. Quercetin was found to inhibit NS3 activity in a specific dose-dependent manner in an *in vitro* catalysis assay, also inhibiting HCV RNA replication in the subgenomic HCV RNA replicon system and virus production in the HCV infectious cell culture system[37]. Gonzalez *et al*[38] have shown the marked reduction in viral production imparted by heat shock proteins synthesis inhibitor Quercetin. The low toxicity and pharmacokinetics of Quercetin are well known, and it has been approved for other uses in clinical trials. In fact, a phase I study evaluating the safety and tolerability of Quercetin in hepatitis C patients who have contraindications to standard antiviral treatment started last year (www.clinicaltrials.gov).

Administration of the exogenous interferons (IFNs) alpha, beta, and gamma in the setting of treatment for chronic HCV infection and other conditions has been shown to lower LDL cholesterol and raise triglyceride levels in VLDL, concomitant with suppression of lipoprotein lipase[39,40]. Li *et al*[41] have shown an association between rs12979860 genotype and host serum lipid levels, suggesting a relationship between endogenous IFN response and lipids. They hypothesize that the IFN-lambda rs12979860 CC responder genotype, which was associated with both increased likelihood of treatment response and higher LDL cholesterol levels in the studied cohort, is associated with lower IFN-lambda activity or lower intrahepatic IFN signaling gene expression. Our results indicate that LDL and total cholesterol levels were higher in patients infected with HCV genotype 1 harbouring the favourable genotype for interleukin 28B gene (Del Campo *et al*[29] unpublished data). These results suggest that observed associations are directly related to HCV-host interactions instead of a direct effect of this locus on lipid metabolism. At least in part, this host factor could select virus infection and promote chronic infection or spontaneous clearance according to viral genotype and lipid metabolism interplay.

Final remarks

HCV entry is a highly orchestrated process involving several viral and host cell factors, affecting infection, spontaneous viral clearance, persistent infection and widespread, and thereby offering multiple novel targets for antiviral therapy. A recently discovered novel surface receptor involved in HCV entry, NPC1L1, is responsible for cellular cholesterol absorption. This receptor arose as a new therapeutic target for HCV infection, since specific antibodies can block HCV entry. Ezetimibe treatment delayed the establishment of HCV infection in an animal model, emphasizing the relevance of the interaction between the host lipid metabolism and the establishment of a persistent infection, although utilized doses could not be translate to clinical practice. Host and virus genetic variation together with the interaction with hepatocyte receptors were assumed to explain the heterogeneity in HCV outcomes across individuals.

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TOPIC HIGHLIGHT

Toru Ishikawa, MD, *Series Editor*

Early administration of branched-chain amino acid granules

Toru Ishikawa

Toru Ishikawa, Department of Gastroenterology and Hepatology, Saiseikai Niigata Daini Hospital, Niigata 950-1104, Japan
Author contributions: Ishikawa T contributed solely to this review.
Correspondence to: Toru Ishikawa, MD, PhD, Department of Gastroenterology and Hepatology, Saiseikai Niigata Daini Hospital, 280-7 Teraji, Niigata 950-1104, Japan. toruishi@ngt.saiseikai.or.jp
Telephone: +81-25-2336161 Fax: +81-25-2338880
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Abstract

The effect of malnutrition on survival in patients with decompensated liver cirrhosis has not been well defined. Nutritional intervention with branched-chain amino acid (BCAA) can increase serum albumin concentration in patients with decompensated cirrhosis but its effects on survival are unclear. The BCAA to tyrosine ratio (BTR) is a surrogate marker (the normal range of BTR is between 4.41 and 10.05, and a Fischer's ratio of 1.8 corresponds to a BTR of 3.5) in patients with decompensated liver cirrhosis, and BCAA inhibits hepatic carcinogenesis in patients with compensated cirrhosis. This review discusses data regarding the effect of early administration of BCAA granules based on the ratio of BCAA to BTR on prognosis in patients with cirrhosis.

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Key words: Branched-chain amino acid to tyrosine ratio; Branched-chain amino acid granules; Liver cirrhosis; Nutritional intervention; Malnutrition; Quality of life; Albumin; Cancer onset

Peer reviewers: Dr. Richard Parker, Department of Gastroenterology, Sandwell and West Birmingham NHS Trust, Dudley Road, Birmingham B1 2AF, United Kingdom; Ilker Tasci, Department of Internal Medicine, Gulhane School of Medicine, GATA Ic Hastaliklari Bilim Dali, 06018 Etlik, Ankara, Turkey

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INTRODUCTION

The liver plays a key role in nutrient metabolism, and patients with cirrhosis may develop various metabolism and nutrition disorders. In fact, many cirrhosis patients suffer from protein-energy malnutrition (PEM)[1], which is particularly pronounced in the decompensated stage. Correction of PEM can improve prognosis in patients with decompensated cirrhosis[2]. Patients with cirrhosis and decreased plasma branched-chain amino acid (BCAA) levels can develop PEM with increased catabolism[3].

PEM is associated with a high morbidity and mortality due to an increased risk of life-threatening complications, resulting in poor survival and poor quality of life (QoL)[4,5].

SIGNIFICANCE OF BCAA SUPPLEMENTATION

BCAAs are a group of essential amino acids, including valine, leucine, and isoleucine. A low plasma level ratio of BCAAs to aromatic amino acids suggests the presence of liver cirrhosis, and BCAA supplementation was originally developed in order to normalize the patient's amino acid profile and nutritional status[6].

This article summarizes the findings of previous studies to determine whether nutritional intervention with a granulated BCAA preparation can contribute to improved prognosis of patients with PEM and cirrhosis. In Japan, oral BCAA preparations are used in nutritional therapy to correct protein and amino acid abnormalities

in patients with cirrhosis. Moreover, enteral nutrition guidelines published by the European Society for Clinical Nutrition and Metabolism list BCAA supplementation as a grade B recommendation in the treatment of advanced cirrhosis[7].

BCAA supplementation effectively increases Fischer's ratio and improves hypoalbuminemia in patients with liver cirrhosis[8]. In addition, not only does BCAA serve as components of albumin, the activation by *L*-leucine of mammalian target of rapamycin in hepatocytes and the subsequent activation of albumin mRNA transcription and protein synthesis in ribosomes are thought to constitute the mechanism for albumin increase[9,10]. This increase in albumin has been verified in various clinical trials to be effective for improving hypoalbuminemia[8,11].

Oral BCAA preparations come in two dosage forms: enteral nutrition formulas (or elemental diet products) for liver failure and oral BCAA granules. In 1996, an oral BCAA granule product containing valine, leucine, and isoleucine (Val, Leu, Ile) in a composition ratio of 1:2:1.2 was marketed in Japan (Livact®, Ajinomoto Pharmaceuticals, Tokyo, Japan). This product is indicated for decompensated cirrhosis patients who have hypoalbuminemia despite adequate dietary intake.

IMPROVEMENT OF PROGNOSIS AND INHIBITION OF HEPATOCAR-CINOGENESIS

An Italian research group reported a multicenter randomized trial in which a total of 174 patients with advanced liver cirrhosis were given BCAA as a supplement for 1 year and its effects were compared with administration of lactalbumin or maltodextrin[12]. Long-term use of a BCAA granule preparation has been reported to increase serum albumin and to inhibit the incidence of events related to poor prognosis[8,12].

Moreover, Marchesini *et al*[12] analyzed whether oral BCAA might prevent progressive liver failure and improve nutritional parameters and quality of life.

They conclude that long-term nutritional supplementation with oral BCAA is useful to prevent progressive hepatic failure and to improve health status ,and recommend that new formulas are needed to increase compliance. An oral BCAA granule (Livact®, Ajinomoto Pharmaceuticals, Tokyo, Japan) is the form of small uniform granules, which reduces BCAA-induced stimulation of taste buds and contributes to improve compliance.

Kobayashi *et al*[13] conducted a study of patients with compensated cirrhosis caused by hepatitis C. After a mean follow-up of 3.2 years, the authors reported that the incidence of hepatocellular carcinoma (HCC) among men with a baseline serum albumin level of 3.6 to 4.0 g/dL tended to be lower in the BCAA granule treatment group than in the control group. A large-scale postmarketing clinical study conducted at 89 sites in Japan to determine the effects of BCAA granules on the prognosis of cirrhosis patients [Long-term Survival Study (LOTUS)] demonstrated that the onset of complications associated with poor prognosis (i.e., liver failure, ruptured esophageal varices, HCC, and death) was significantly lower among patients in the BCAA granule treatment group than in the dietary therapy group (hazard ratio: 0.67; 95% CI: 0.49-0.93)[8]. Furthermore, stratified analysis of the LOTUS study for groups at high risk for HCC, specifically, patients with a body mass index $\geqslant$ 25 kg/m^2 or elevated alpha-fetoprotein, showed that BCAA granules had an inhibitory effect on the incidence of HCC[14]. Meanwhile, a study by Tsuchiya *et al*[15] on radical therapy for HCC patients reported that long-term treatment with BCAA granules reduced the rate of the third and subsequent relapses of HCC and improved the cumulative rate of survival in patients with baseline serum albumin $\leqslant$ 3.5 g/dL. Sato *et al*[16] undertook a comparative study of dietary control protocols and found that a BCAA granule product and an enteral nutrient for liver failure (or an elemental diet product) had similar effects in terms of improving or maintaining serum albumin and preventing the onset of hepatic encephalopathy. The authors reported that, despite control of the total dietary energy intake, an increase in glycosylated hemoglobin and other markers of abnormal glucose tolerance occurred in the enteral nutrient group, whereas these unfavorable changes were not observed in the BCAA granule treatment group[16].

Moreover, Hayaishi *et al*[17] reported that Oral BCAA supplementation is associated with reduced incidence of HCC in patients with cirrhosis.

Based on these findings, a BCAA granule preparation (Livact®) has been now recommended in the guidelines for the treatment of liver cirrhosis by the Study Group for the Standardization of Treatment of Viral Hepatitis Including from the Ministry of Health, Labour and Welfare in Japan in order to increase serum albumin in cirrhosis patients with the aim of reducing the onset of cancer[18] (Table 1).

IMPORTANCE OF CONTINUED ADMINISTRATION

On the other hand, because some patients do not exhibit any increase in serum albumin after taking BCAA granules, further research has been conducted on the dietary intake and baseline characteristics of these patients. Yatsuhashi *et al*[19] reported that the anti-hypoalbuminemic effect of BCAA granules was not influenced by dietary intake and that continued use of BCAA significantly reduced the incidence of ascites and edema even in patients whose serum albumin did not respond to BCAA granule treatment.

One possible explanation for the significant decline in ascites and edema in the unchanged serum albumin group could be that the BCAA granules improved albumin quality. The sulfhydryl group of the cysteine 34 residue in human serum albumin can exist in a reduced state (reduced albumin) or oxidized state (oxidized albumin). In patients with chronic liver disease, however, the pro-

Table 1 Studies of outcome by branched-chain amino acid administration for cirrhotic patients

Authors	No. of cases	Study time	Outcome
Muto *et al*[8]	646	2 yr	Improving event-free survival, serum albumin concentration, and QoL
Kobayashi *et al*[13]	40	168 wk	Inhibiting hepatic carcinogenesis
Muto *et al*[14]	646	2 yr	Reducing the risk for liver cancer
Fukushima *et al*[21]	7	8 wk	Improving the oxidized/reduced state of serum albumin

QoL: Quality of life .

portion of oxidized albumin increases as the condition progresses[20]; this is associated with body fluid retention, such as ascites and edema. Furthermore, BCAA granules reduce the ratio of oxidized albumin in decompensated cirrhosis patients[21]. These findings suggest that the use of BCAA granules is important in maintaining serum albumin and that its continued use can improve the prognosis of patients with decompensated cirrhosis[22-25].

BCAA TO TYROSINE RATIO VALUE, AN IMPORTANT INDICATOR IN EARLY ADMINISTRATION OF BCAA GRANULES

However, the therapeutic effects of BCAA granules can take longer to appear in patients with advanced decompensated cirrhosis, making it important to determine the optimal timing for administration. After examining the biochemical test results of decompensated cirrhosis patients, Kato *et al*[26] identified the following four characteristics of uncompensated cirrhosis, and thus, the proper time to begin administration of BCAA granule preparations: (1) serum albumin $\leqslant$ 3.5 g/dL; (2) BCAA to tyrosine ratio (BTR) $\leqslant$ 3.5; (3) prothrombin activity $\leqslant$ 60%; and (4) platelet count of $\leqslant$ 100 000/mm^3. This in turn led to the search for markers of cirrhosis in order help facilitate determination of the optimal timing to initiate treatment with BCAA granules.

Cirrhosis patients exhibit shifts in their plasma free amino acid concentration, marked declines in BCAA (Val, Leu, Ile), and increases in aromatic amino acids (AAA; tyrosine, phenylalanine) and methionine, whereas their Fischer's ratio (ratio of molar concentrations of BCAA: AAA) or BTR declines in conjunction with disease severity. Fischer's ratio has long been used to analyze plasma free amino acids[27], but the BTR is a simpler method. Azuma *et al*[28] reported that the BTR based on an enzymatic method serves as an alternative to Fischer's ratio and is a potential indicator of liver disorders as well as subsequent chronic liver disease progression.

In the event of malnutrition, the BTR also declines before the serum albumin declines; therefore, determining the BTR is useful for the early detection of potential hypoalbuminemia. In other words, calculating the BTR enables the prediction of serum albumin level changes[29] and therefore allows determination of the appropriate time to administer BCAA granules. Given this time lag between decreases in serum albumin and BTR, monitoring of BTR needs to be done separately from that of albumin when considering prognostic factors for decompensated cirrhosis. The benefits of administering an oral BCAA preparation in patients with decreased BTR have already been reported in a large-scale clinical study[8]. This also implies that the BTR has considerable potential as a prognostic factor of HCC in decompensated cirrhosis patients. In fact, many reports performed the usefulness of BCAA for the treatment of hepatocellular carcinoma[30-39]. So, BTR may be useful as an indicator of prognosis in patients with HCC[40]. Early administration of BCAA granules based on the ratio of BCAA to tyrosine can improve the prognosis of decompensated cirrhosis.

In conclusion, BCAA supplementation for liver disorders may be expected not only to increase serum albumin, but also to exert other effects, such as prolongation of survival among liver cirrhosis patients, prevention of liver cancer, and enhancement of QoL. However, in cases of severe decompensated liver cirrhosis, determination of the timing of administration is also an important issue because BCAA granules take time to take effect. The decrease in BTR precedes reductions in serum albumin. While early therapeutic intervention with BCAA granules can help improve the prognosis of patients with decompensated cirrhosis and low BTR, more research and analysis are needed to fully explore the novel effects of BCAA granule preparations.

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GUIDELINES FOR CLINICAL PRACTICE

Donation after cardio-circulatory death liver transplantation

Hieu Le Dinh, Arnaud de Roover, Abdour Kaba, Séverine Lauwick, Jean Joris, Jean Delwaide, Pierre Honoré, Michel Meurisse, Olivier Detry

Hieu Le Dinh, Arnaud de Roover, Pierre Honoré, Michel Meurisse, Olivier Detry, Department of Abdominal Surgery and Transplantation, University Hospital of Liège, University of Liège, 4000 Liège, Belgium
Abdour Kaba, Séverine Lauwick, Jean Joris, Department of Anesthesia and Intensive Care Medicine, University Hospital of Liège, University of Liège, 4000 Liège, Belgium
Jean Delwaide, Department of Hepatology and Gastroenterology, University Hospital of Liège, University of Liège, 4000 Liège, Belgium
Author contributions: Le Dinh H performed the literature review and wrote the manuscript; de Roover A, Kaba A, Lauwick S, Joris J, Delwaide J, Honoré P and Meurisse M constitute the team involved in the care of the liver transplant patients and they reviewed and commented the manuscript; Detry O supervised the review.
Correspondence to: Olivier Detry, Professor, Department of Abdominal Surgery and Transplantation, University Hospital of Liège, University of Liège, Sart Tilman B35, 4000 Liège, Belgium. oli.detry@chu.ulg.ac.be
Telephone: +32-4-3667645 Fax: +32-4-3667069
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Abstract

The renewed interest in donation after cardio-circulatory death (DCD) started in the 1990s following the limited success of the transplant community to expand the donation after brain-death (DBD) organ supply and following the request of potential DCD families. Since then, DCD organ procurement and transplantation activities have rapidly expanded, particularly for non-vital organs, like kidneys. In liver transplantation (LT), DCD donors are a valuable organ source that helps to decrease the mortality rate on the waiting lists and to increase the availability of organs for transplantation despite a higher risk of early graft dysfunction, more frequent vascular and ischemia-type biliary lesions, higher rates of re-listing and re-transplantation and lower graft survival, which are obviously due to the inevitable warm ischemia occurring during the declaration of death and organ retrieval process. Experimental strategies intervening in both donors and recipients at different phases of the transplantation process have focused on the attenuation of ischemia-reperfusion injury and already gained encouraging results, and some of them have found their way from pre-clinical success into clinical reality. The future of DCD-LT is promising. Concerted efforts should concentrate on the identification of suitable donors (probably Maastricht category Ⅲ DCD donors), better donor and recipient matching (high risk donors to low risk recipients), use of advanced organ preservation techniques (oxygenated hypothermic machine perfusion, normothermic machine perfusion, venous systemic oxygen persufflation), and pharmacological modulation (probably a multi-factorial biologic modulation strategy) so that DCD liver allografts could be safely utilized and attain equivalent results as DBD-LT.

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Key words: Non-heart-beating donation; Complication; Bile duct; Allocation; Ischemia; Ischemia-reperfusion injury; Liver disease

Peer reviewers: Bijan Eghtesad, Associate Professor, Department of General Surgery, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, United States; Tokihiko Sawada, Associate Professor, Second Department of Surgery, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan; Philip Rosenthal, Professor, Pediatrics, UCSF, 500 parnassus Avenue, San Francisco, CA 94143-0136, United States

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INTRODUCTION

The first human liver transplantations (LT) were performed from donation after cardio-circulatory death (DCD) in the 1960s[1-4]. DCD-LT was nonetheless almost universally abandoned in the following two decades, given the well-recognized Harvard brain-dead concept in 1968 and given the better results of LT originating from donation after brain death (DBD)[5]. In 1983, LT was approved as a therapeutic modality for end-stage liver diseases after a long period considered as an experimental procedure. The renewed interest in DCD donors started in the 1990s following the limited success of the transplant community to expand the DBD organ supply and following the request of potential DCD families.

If DCD kidneys are increasingly accepted around the world[6], the use of DCD livers remains limited in experienced transplant centers due to higher risks of primary graft dysfunction and biliary complications as well as a lack of a reliable viability testing prior to liver implantation. However the number of DCD-LT increased rapidly over the past decade. In the United States, 276 DCD liver transplants were performed in 2008 compared to only 23 cases in 1999, making up 5% of the deceased donor (DD) liver transplants[7-10]. The same trend was observed in the United Kingdom[11-13], Spain[14], Netherlands[15] and Belgium[15,16]. Netherlands had the highest rate of DCD- over DD-LT in the world (22.5% in 2008)[15]. France has just initiated its DCD-LT program since 2010[17]. In Japan, although DCD donors were the essential DD source, its use was reserved mainly for kidney, pancreas and islet transplantation[18]. Using a mathematical model to analyze the potential impact of a DCD policy on LT programs, Chaib *et al*[19] reported if 1%, 5% and 10% of deceased individuals became DCD donors, there would be 8%, 27% and 37% relative reductions in the size of waiting list, respectively. The use of DCD livers could increase the supply of transplants by 53%[20]. Centers with active DCD-LT programs usually reported 4%-10% rates of LT from the DCD source[21]. The potential impact of DCD use on the DBD availability is also a controversial issue. Controlled DCD programs might negatively influenced DBD activity in Belgium, Netherlands and United Kingdom while uncontrolled DCD donors seemed to be a clear additional source of organs for transplantation in France and Spain[22].

Most countries use Maastricht-category-3 DCD donors for LT, except France and Spain, where categories 1 and 2 are exclusively used due to legal interdiction of discontinuation of therapy in irreversibly brain-injured individuals[17,23,24]. German law prohibits any DCD organ procurement and transplant activity. In Italy, death of a human being must be declared 20 min after cardiac arrest using continuous electrocardiography. The procedure therefore will enable, at best, retrieval of only a few marginal kidneys and some tissues, and will not be helpful for patients on LT waiting lists[25]. This article is aimed at reviewing mono- and multi-centric DCD-LT outcomes, experimental strategies on animal models to optimize the utilization of this donor source and its future development.

DIFFERENCES BETWEEN DCD AND DBD DONORS PERTINENT TO LT OUTCOMES

Generally results of DCD-LT are inferior to those from DBD-LT with regard to both short-and long-term graft and patient survival as well as post-transplant morbidity. Expected DCD-LT outcomes could be explained by inherent differences between DCD and DBD donors in circumstances of death, warm ischemia time (WIT) and donor cause of death. Consequently, a different strategy of DCD use in terms of logistics of organ retrieval and preservation, allocation and recipient selection appears necessary to guarantee acceptable results. These differences will be briefly discussed prior to considering results of DCD-LT in detail.

Circumstances of death and consequent warm ischemia time

In DCD, donor death is diagnosed on the basis of irreversible cessation of cardio-pulmonary function instead of conventional neurologic criteria. As a result, organs from DCD donors are subjected to a period of hypotension, hypoxia and a circulation prior to organ procurement and this WIT adversely affects tissue viability and graft function after transplantation[26]. An international classification of DCD donors into 4 categories was first proposed in 1995 and widely accepted up to now[27]. New DCD categories have been recently suggested in Spain[28,29], Italy[30] and Belgium[31]. The length of WIT varies greatly according to the type of DCD process. It is longest among uncontrolled categories 1 and 2 (usually 90-120 min) and shorter among controlled categories 3 and 4 DCD donors (usually 20-30 min). In brain death, issues related to donor warm ischemia are eliminated because DBD donors have an effective natural organ perfusion and a potentially well-preserved organ function and WIT is thus nearly equal to zero.

However, WIT is heterogeneously defined among authors[32]. In the controlled DCD context, the commonest definition is the time interval between withdrawal of both ventilator and cardiac support to start of cold flushing of the organ[33,34]. This definition includes the no-touch period and the time of death declaration and is proposed to have two phases (withdrawal and acirculatory phases). Other authors used a blood pressure (BP) or oxygen saturation threshold below which would be defined as the beginning of true WIT (systolic or mean BP < 35-60 mmHg, oxygen saturation < 25%-70% or unreadable)[35-42]. de Vera *et al*[34] did not use a BP threshold to define the start of WIT because tissues are still hypoxic in a DCD donor who maintains a BP but has ceased to ventilate. It is unknown at what BP or oxygen saturation the liver parenchyma and biliary system undergo irrecoverable injury[43]. The first international Non-Heart Beating Donor workshop in Maastricht in 1995

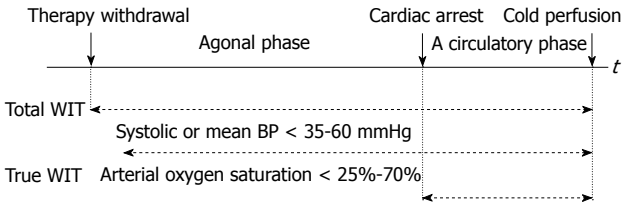


Figure 1 Different ways of warm ischemia time definition in the controlled donation after cardio-circulatory death setting (see text for more details). True warm ischemia time (WIT) is also called complete or functional WIT; Total WIT is also called overall WIT; Agonal phase is also called withdrawal phase. BP: Blood pressure; *t*: Time.

suggested WIT should be calculated from the moment of cardiac arrest until the start of hypothermic flush-out[44]. This definition may be useful for consistency but is inaccurate at the cellular level. Hypoxia starts when the blood flow or oxygenation no longer meets cellular metabolic needs[37]. The start of WIT may be chosen prior to asystole, and the end of WIT may be at or after aortic flushing[45]. Apparently a well-accepted definition of donor organ ischemic times is needed to standardize nomenclature and allow accurate comparisons of individual DCD studies[46,47] (Figure 1).

In transplant practice, WIT should be minimized as much as possible. For controlled DCD donors, the possibility to predict whether a potential donor will or will not expire in a time frame consistent with donation is extremely important, because prolonged time to asystole, likely resulting in suboptimal organ perfusion, is a common reason for non procurement of DCD grafts[48,49]. Time between therapy withdrawal and cardiac arrest usually does not exceed 1 h in most DCD donors. However, if a DCD donor has a period of relatively hemodynamic stability after life-support withdrawal, this period may be extended beyond 1 h without additional warm injury to the organs[50]. Some authors emphasized during the withdrawal phase, time to a systolic BP < 50 mmHg should be < 30 min[20] and the hypotensive period (mean BP < 50 mmHg) < 15 min[51]. Manara *et al*[52] proposed the so-called functional WIT, which is measured from the donor's systolic BP < 50 mmHg, the arterial oxygen saturation < 70%, or both, to the start of cold perfusion, should not exceed 30 min and may be limited to 20 min in suboptimal donors. Several factors have been identified as predictors of rapid death following treatment withdrawal and include the DCD tool of University of Wisconsin[53], donor Glasgow coma scale, inotropic use, BP at treatment discontinuation, high FiO_2 and mode of ventilation[54,55]. Withdrawal of therapy is preferably occurred in the operating room with a donor surgical team immediately available. Prior to cessation of the ventilator and organ perfusion support, the donor may be already prepared and draped, and the surgical instruments, preservation solution and tubing are set up to facilitate rapid organ recovery. The super rapid recovery technique is preferable and organs may be removed *en bloc*[39,50]. For uncontrolled donors, *in vivo* organ preservation techniques, like in-situ intravascular cooling using a double balloon and triple lumen catheter or hypo- and normothermic cardiopulmonary bypass with extracorporeal membrane oxygenation (ECMO), should be employed. With regard to the logistic organization, two frequently mentioned initiatives are the "Maastricht's box" and the "Madrid's rapid identification and response system"[6].

Donor cause of death

DCD donors do not experience the brain dead process. Brain death provokes a cascade of changes in hemodynamics, hormones, and immune response, which negatively affect donor organ viability and transplant outcomes[56,57]. Hemodynamic instability may have deleterious effects on liver function, although the liver has a high tolerance to marked hypotension and a large physiological reserve. Only a few histological changes were observed in the liver both on light and electron microscopic examination during the brain dead process[58,59]. The most important changes are the increased liver immunogenicity with subsequent increased host alloresponsiveness and the occurrence of apoptosis of hepatocytes[60]. Clinical findings in livers from DBD donors revealed significantly higher leukocyte infiltrates, up-regulation of adhesion molecules [intercellular adhesion molecule (ICAM), vascular cell adhesion molecule] and pro-inflammatory cytokines [interleukin-6 (IL-6), IL-10, IL-1β, interferon γ and tumor necrosis factor-alpha (TNF-α)], along with an increased expression of major histo-compatibility complex-Ⅱ relative to livers from living donors[61,62]. The peak time of cytokine expression and cell infiltration is during brain death and organ procurement but not after reperfusion[61]. These changes may amplify ischemia-reperfusion injury (IRI) during the transplant procedure and accelerate graft rejection after transplant[63]. In reality, donor brain-death mechanisms are quite varied and large differences may exist in the degree of impaired organ quality and transplant outcomes. The impact of donor cause of death on transplant outcomes has been recently confirmed in a United Network for Organ Sharing (UNOS) registry analysis, in which the cerebro-vascular accident presented as a predictor of worse graft survival across all organs relative to other donor modes of death[64].

Uncontrolled DCD donors whose cause of death is usually other than neurologic do not undergo the process of brain death, while most controlled DCD donors have sustained irreversible cerebral injuries. As a result, organs from controlled DCD donors are likely to suffer more from the harmful immunologic and inflammatory effects of acute brain injury than those from uncontrolled DCD donors[65].

Allocation policy

It is reported that organs that have already subjected to warm ischemic injury have an increased susceptibility to damage during cold storage[66]. The incidence of primary non-function (PNF) was 2.5 times less in patients with

Table 1 Risk classification for donation after cardio-circulatory death donors and donation after cardio-circulatory death-liver transplantations recipients

Authors		Donors	Recipients
Mateo *et al*[74]	Low risk	Both WIT ⩽ 30 min and CIT ⩽ 10 h	RCRR ⩽ 1.5
	High risk	WIT > 30 min and/or CIT > 10 h	RCRR > 1.5 Re-transplantation and/or On life-support and/or A combination of ⩾ 3 risk factors: Hospitalization or in an intensive care unit Serum creatinine > 2 mg/dL On dialysis Age > 60 yr
Lee *et al*[32]	Low risk	Donors with no identified donor risk factors	Recipients with no identified recipient risk factors
	High risk	Donors with at least one identified donor risk factor: Donor age > 45 yr WIT > 15 min CIT > 10 h	Recipients with at least one identified recipient risk factor: Previous transplantation Life support at transplantation
de Vera *et al*[34]	Low risk	-	MELD scores ⩽ 30
	High risk	-	MELD scores > 30 On life support (mechanical ventilation, hemodialysis)

RCRR: Recipient cumulative relative risk; WIT: Warm ischemia time; CIT: Cold ischemic time; MELD: Model for end-stage liver disease.

cold ischemia time (CIT) ⩽ 8 h *vs* those with CIT > 8 h (5% *vs* 13%)[34]. The incidence of graft failure within 60 d of transplantation was 10.8% if CIT < 8 h and substantially increased to 30.4% and 58.3% if CIT > 8 h and > 12 h, respectively[67]. Proper and rapid allocation of DCD livers thus appears pivotal to minimize CIT. One-year graft survival of DCD livers shared regionally was less good than those shared locally (67% *vs* 77%)[68] and the relative risk of graft failure from nationally shared DCD livers was 31% higher than locally or regionally shared ones[69]. Thus a policy to favor local use of DCD livers seems reasonable[67,68]. However, parallel (backup) offers should also be made to expedite organ placement[33]. The exchange of DCD livers between transplant centers has been successfully done but requires a more efficient and rapid referral system due to a lower tolerance of these allografts to cold storage[70].

Regarding recipient selection criteria, DCD livers could be routinely discussed and offered to all recipients on the waiting list[20,70,71] or selectively reserved to uncomplicated cases to ensure short CIT (by avoiding cases with extensive history of abdominal surgery or portal-vein thrombosis)[20,35]. An expected long surgical procedure exceeding 8 h of CIT, logistical reasons for an extended CIT, combined organ transplantation, recipients with high Model for End-Stage Liver Disease (MELD) scores or a large age difference between donors and recipients could all result in the refusal of a DCD liver[51]. Patients with stable cholestatic liver disease or re-transplantation were also excluded from DCD programs because of problems related to the quality of life in primary biliary cirrhosis and to the fear that pre-existent warm ischemic biliary damage could trigger the recurrence of primary sclerosing cholangitis[72]. Using DCD livers in re-transplanted patients might increase the CIT associated with a difficult hepatectomy. Recently LaMattina has demonstrated the feasibility of simultaneous liver and kidney (SLK) transplantation using DCD donors and shown short-term results comparable to those of SLK transplantation using DBD donors, making it a valid approach to safely expanding the donor organ pool for patients with end-stage liver and kidney disease[73].

It is still controversial whether it is better to transplant such grafts into healthy or sicker recipients (i.e., according to the recipient liver disease severity). UNOS database reviews advocated utilizing DCD livers in "low-risk" recipients[32,67,74]. de Vera *et al*[34] also observed better graft survival when DCD livers were utilized in patients with MELD scores ⩽ 30, but simultaneously could demonstrate that "sicker", high-risk recipients (at MELD scores > 30 or on organ-perfusion support, like mechanic ventilation or hemodialysis) had a greater patient and graft survival benefit from the transplantation of DCD livers compared to patients who are not as critically ill. Risk classification for DCD donors and DCD-LT recipients is summarized in Table 1. Other groups of patients that may have a true survival benefit from DCD-LT include MELD "disadvantaged" patients (hepato-cellular carcinoma patients beyond the Milan criteria or who are listed in areas with long waiting times, patients with low MELD scores that do not adequately reflect their level of illness and their critical need for a transplant)[34,72].

Studies about the effect of DCD liver grafts on hepatitis-C virus positive (HCV+) recipients transplant outcomes were inconsistent. Nguyen *et al*[45] and recently Hernandez-Alejandro *et al*[75] found a negative effect of HCV on DCD livers, but a formal contraindication for the use of DCD liver allografts in HCV+ recipients was not justified except for older donors. In fact, while single-center series reported no significant difference in graft and patient survival rates of HCV+ recipients and graft loss from HCV recurrence between DCD and DBD groups[20,34,76,77], as well as no deleterious effects of DCD liver grafts on the disease progression (fibrosis)

in comparison with DBD liver grafts in HCV+ recipients[77], the most recent UNOS registry data showed inferior graft survival but similar patient survival of HCV+ recipients with DCD donors compared to ones with DBD donors. Furthermore, DCD livers on HCV disease do not fare worse than DCD livers on non-HCV disease. DCD livers thus appeared to be important source of LT for HCV patients[78]. Split livers from DCD donors have also been reported in recent years with acceptable results[79,80].

TRANSPLANT OUTCOMES

Currently one-year patient survival after DBD-LT and to a certain extent after controlled DCD-LT is about 85%-90% in comparison to 60% in the early eighties and around 30% in the early days of LT and at 5 years post-transplant patient survival rate remains over 70%. Medical progress over the past 40 years in the field of organ preservation, surgical techniques, immunosuppressive drugs, treatment of post-transplant complications and organ allocation has permitted DCD to become reality in the modern era. Although there are concerns about the quality of such organs, with evidence that a prolonged WIT causes a raised incidence of PNF and biliary complications as well as suboptimal graft and patient survival when compared to DBD livers, DCD livers may be life-saving for those who would die waiting for a DBD liver[68] and do increase the number of organs available for LT. With careful donor/recipient selection and matching, minimization of ischemia and good post-operative care, acceptable results can be achieved. Essential results of most important publications in the last decade in DCD-LT are presented in Tables 2-4.

PNF

PNF is usually defined as unrecoverable hepato-cellular dysfunction leading to patient death or re-transplantation within the first week post-transplant after excluding other causes of graft failure such as vascular thrombosis, biliary complications, rejection or recurrent disease[81-84]. Initial studies using uncontrolled DCD donors reported a rate of PNF as high as 50%[85]. Currently only a few transplant centers in the world (like Spain, France) used this kind of donors because of aforementioned reasons. By using different *in vivo* organ preservation methods to maintain DCD donors and by strictly applying donor selection criteria, authors in Madrid[71], Barcelona[29] and La Coruña[86-89] could obtain promising results from Maastricht category I and II donors with a PNF rate of 10%-25%. The discard rate nevertheless was high up to 50%-75%[29,71]. In controlled DCD donors, the PNF rates are 0% to 12%. Matched analysis[34,72] and registry data[67,68] showed a higher rate of PNF in controlled DCD than DBD donors, although no difference was found in most comparative studies[20,43,90,91] except one[92]. The increased risk of PNF in DCD-LT recipients was also confirmed in a recent meta-analysis (odds ratio = 3.6, 95% CI: 2.1-6.4)[93]. Case-series reports of controlled DCD-LT also had a rate of PNF between 0% and 10%[42,70,94-97].

PNF is the consequence of severe IRI with the initial period of warm ischemia playing a crucial role. Experimental evidence supported that donor WIT should be less than 30 min to minimize PNF[98]. This warm ischemia (WI) period increases graft susceptibility to damage during cold preservation and CIT was a main contributing factor to PNF[34,67]; therefore, both periods of ischemia must be kept to a minimum. Many laboratory tests have been developed both in animal models and in human to predict the probability of occurrence of PNF post-transplant, but none is yet clinically efficient[99]. Recently Dahaba *et al*[100] proposed bispectral index monitoring as an early intra-operative indicator of early graft dysfunction.

Biliary complications

Since the introduction of LT up to now, biliary complications are always regarded as the "Achilles heel" and a major cause of morbidity and graft failure in patients after LT[101]. The most common biliary complications are bile leakage and bile duct stricture[102,103]. Strictures involving the donor bile duct (> 1 cm above the biliary anastomosis) and requiring endoscopic or radiological dilatation/stenting or surgery in the face of a patent, non-stenotic hepatic artery was referred to as ischemic-type biliary lesions (ITBL), based on the radiologic resemblance of those occurring after hepatic artery thrombosis (HAT)[51,91,103].

Abt *et al*[104] first mentioned the significantly higher incidences of overall biliary complications as well as ITBL in DCD-LT recipients, the finding which was later confirmed in both matched[34,72] and comparative[20,43,51,89,91,104] studies except series of Fujita *et al*[105] and Manzarbeitia *et al*[106]. The rates of overall biliary complications and ITBL were 10.5%- 53% and 8.3%-38%, respectively in DCD-LT compared to 8.3%-22% and 0%-8%, respectively in DBD-LT. Especially Jiménez-Galanes *et al*[71] reported only a 5% incidence of ITBL in their patients receiving livers from uncontrolled DCD donors under normothermic ECMO. A recent meta-analysis revealed that DCD recipients had a 2.4 times increased odds of biliary complications (95% CI: 1.8-3.4) and a 10.8 times increased odds of ITBL (95% CI: 4.8-24.2) *vs* DBD recipients. In average, biliary complications were present in 29% of DCD compared with 17% of DBD recipients and ITBL in 16% of DCD *vs* 3% of DBD recipients[93].

Furthermore DCD recipients who developed ITBL experienced a fairly rapid clinical deterioration, characterized by a relatively short mean time from transplant to first endoscopic retrograde percutaneous cholangiopancreatography (ERCP), from first ERCP to relisting and from relisting to re-transplantation (within 180 d)[36,69]. ITBL results in re-operation, multiple endoscopic and percutaneous biliary interventions, re-transplantation and even patient death with markedly increased medical care costs[107]. The relative risk (RR) of developing graft loss with ITBL formation was 3.02 (95% CI: 1.9-5.3)

Table 2 Results of donation after cardio-circulatory death-liver transplantations in single-center studies

Authors study period	Transplant center	Publication year	Patient number and Maastricht category	WIT (min)	CIT (min)	Mean follow-up	PNF %	Major biliary complications %	ITBL %	HAT %	HAS %	Rejection %	Retransplantation %	Graft survival %				Patient survival %			
														1 yr	3 yr	5 yr	10 yr	1 yr	3 yr	5 yr	10 yr
Casavilla *et al*[85]	Pittsburgh,	1995	6 DCD_4	37	10.6 h	-	50[1]	-	-	16.6	-	-	83.3	17	-	-	-	67	-	-	-
1989-1993	United States		6 DCDc	23.8	11 h	-	0[1]	-	-	33.3	-	-	33.3	50	-	-	-	50	-	-	-
Otero *et al*[87]	Madrid, Spain	2003	20 DCD_2	10	647	> 2 yr	25[1]	30[1]	-	0	-	27	25	80	-	-	-	80	-	-	-
1995-2000			40 DBD	8	405	-	3[1]	8[1]	-	-	-	34	5	55	-	-	-	83	-	-	-
Quintela *et al*[86]	Spain	2005	9 DCD_2 + 1 DCD_4	80	561	-	10	-	-	-	-	-	10	100	-	-	-	100	-	-	-
1995-2004																					
Suárez *et al*[89]	Spain	2008	27 DCD_2	13	635	> 3 mo	18[1]	41.7[1]	25.0[1]	3.6	-	17.4	-	-	-	49[1]	-	-	-	62	-
1994-2005			471 DBD	7	-	-	3[1]	16.8[1]	2.3[1]	3.1	-	28.6	-	-	-	68[1]	-	-	-	74	-
Fondevila *et al*[29]	Barcelona, Spain	2007	10 DCD_1	-	399	-	10	10	-	10	-	-	20	50	-	-	-	70	-	-	-
2002-2006			20 DBD	-	-	-	0	0	-	5	-	-	5	75	-	-	-	80	-	-	-
Jiménez-Galanes *et al*[71]	Madrid, Spain	2009	20 DCD_2	12	432	360 d	10	5	50	0	-	-	1[1]	80	-	-	-	85.5	-	-	-
2006-2008			40 DBD	6	409	-	2.5	-	-	-	-	-	50[1]	87.5	-	-	-	87.5	-	-	-
Pine *et al*[72]	St. James, London,	2009	39 DCDc	13.4	352	2.5 yr	5.1	33.3[1]	20.5[1]	2.6	12.8[1]	20.5	7.6[1]	79.5[1]	63.6[1]	-	-	80[1]	68.2[1]	-	-
2002-2008	United Kingdom		39 DBD	-	593	6.6 yr	-	10.2[1]	-	5.1	-	23.1	2.5[1]	97.4[1]	97.4[1]	-	-	100[1]	1[1]	-	-
de Vera *et al*[34]	Pittsburgh,	2009	141 DCDc	19.8	657	-	12[1]	25[1]	16.3[1]	66	-	-	18[1]	69[1]	-	56[1]	44[1]	79	-	70	57
1993-2007	United States		282 DBD	-	636	-	3[1]	13[1]	< 1[1]	-	-	-	7[1]	82[1]	-	73[1]	63[1]	85	-	76	64
Yamamoto *et al*[90]	Stockholm, Sweden	2010	24 DCDc	6	7 h	> 20 yr	8.3	37.5[1]	-	33.3[1]	-	70.8	-	54.2	-	37.5	37.5	61.9	-	42.9	42.9
1984-1988			16 DBD	-	6.8 h	> 20 yr	18.7	6.3[1]	-	-	-	56.2	-	43.8	-	37.5	37.5	63.6	-	-	54.5
Fujita *et al*[105]	Gainesville, Floria,	2007	24 DCDc	12.8	7.6 h	-	2.8	25	12.5	8.3	-	39.1	20.8	69.1	58.6	-	-	86.8	81.7	-	-
1990-2006	United States		1209 DBD	-	8.1 h	-	-	20.5	-	4.1	-	-	9.4	78.7	70.2	-	-	84	76	-	-
Foley *et al*[91]	Wisconsin,	2005	36 DCDc	17.8	8.2 h	3 yr	5.5	33[1]	13.8	5.5	16.6[1]	61	19.4[1]	67[1]	56[1]	-	-	80[1]	68[1]	-	-
1993-2002	United States		553 DBD	-	8.3 h	4.6 yr	1.3	10[1]	8	11.8	5.4[1]	56	7[1]	86[1]	80[1]	-	-	91[1]	84[1]	-	-
Manzarbeitia *et al*[106]	Philadelphia,	2004	19 DCDc	19.6	574	1000 d	5.2	10.5	-	-	-	-	10.5	-	-	-	-	89.5	-	-	-
1995-2002	United States		311 DBD	-	557	-	-	13.8	-	-	-	-	8.7	-	-	-	-	84.2	-	-	-
Abt *et al*[104]	Pennsylvania,	2003	15 DCDc	20.4	366	819 d	6.7	33.3[1]	26.7[1]	3.2	-	20	6.6[1]	71.8	71.8	-	-	79	79	-	-
1996-2001	United States		221 DBD	-	464	690 d	3.6	9.5[1]	2.3[1]	-	-	21.3	3.6[1]	85.4	73.9	-	-	90.9	77.7	-	-
Nguyen *et al*[45]	Mayo Clinic, Floria,	2009	19 DCDc	16	6.7 h	> 4.5 yr	5.3	26.3	10.5	0	5.3	5.3[1]	15.8	73.7	68.4	63.2	-	89.5	89.5	89.5	-
1998-2001	United States		234 ECD	-	7.1 h	-	4.7	22.6	-	-	-	33.3[1]	8.5[1]	-	-	-	-	85	78.6	72.3	-
			214 SCD	-	7.5 h	-	1.7	15.9	-	-	-	33.2[1]	19.6[1]	-	-	-	-	84.3	80.7	76.5	-

DCDc: Controlled donors after cardiac death; DCD_1, DCD_2 and DCD_4: Maastricht category-1, category-2 and category-4 DCD donors; DBD: Donors after brain death; SCD: Standard criteria donors; ECD: Extended criteria donors; WIT: Warm ischemia time; CIT: Cold ischemia time; PNF: Primary non-function; ITBL: Ischemic-type biliary lesions; HAT: Early hepatic artery thrombosis; HAS: Early hepatic artery stenosis. Major symptomatic biliary complications include biliary leak, anastomotic and non-anastomotic stenosis. [1]Numbers denote the statistically significant difference between groups.

and graft survival was significantly decreased in patients with non-anastomotic strictures, compared to patients without it[89]. Up to 50% of all occurrences of ITBL lead to death and/or re-transplantation[108].

ITBL is usually a reflection of severe IRI in relation to various factors. In animal models, irreversible biliary tract damage has been observed after 40 min of cardiac arrest although hepato-cellular function could be preserved[109]. Clinical observations showed that total WIT > 30 min and chaotic donor physiology before asystole may increase the risk of post-transplant biliary stricture[33,110]. The mechanism could come from the stasis of blood and clot formation in the peri-biliary micro-circulation whose blood is solely supplied by the hepatic artery[96]. Many multivariate analysis recognized DCD liver grafts as an independent risk factor for the appearance of ITBL (RR = 47.1)[51,89].

Table 3 Results of donation after cardio-circulatory death- liver transplantations in single-center studies

Authors study period	Transplant center	Publication year	Patient number and Maastricht category	WIT (min)	CIT (min)	Mean follow-up	PNF %	Major biliary complications %	ITBL %	HAT %	HAS %	Rejection %	Retransplantation %	Graft survival %			Patient survival %		
														1 yr	3 yr	5 yr	1 yr	3 yr	5 yr
Grewal *et al*[20]	Mayo Clinic, Floria,	2009	108 DCDc	22.3	6.3 h	-	3.7	-	8.3[1]	0.9	-	-	14.8	79.3	74.5	71	91.5	88.1	88.1
1998-2006	United States		1328 DBD	-	7.1 h	-	1.4	-	1.9[1]	1.7	-	-	9.3	81.6	74.7	69.1	87.3	81.1	77.2
Kaczmarek *et al*[41]		2007	11 DCDc	34.6	7.6 h	> 14 mo	0	45.4[1]	1[1]	0	-	-	9.1[1]	-	-	-	-	-	-
1999-2006			164 DBD	-	-	-	-	16.4[1]	8.2[1]	-	-	-	0[1]	-	-	-	-	-	-
Dubbeld *et al*[51]		2010	55 DCDc	16.5	456	-	2	28[1]	24[1]	74.7	-	-	18	74	68	-	85	80	-
2001-2006			471 DBD	-	515	-	1.5	8.3[1]	7.9[1]	-	-	-	10.4	80.4	74.5	-	86.3	80.8	-
Chan *et al*[43]	Seattle, United States	2008	51 DCDc	-	-	3 yr	0	23.5[1]	13.7[1]	4.8	-	-	9.8	79	79	-	83	83	-
2003-2006			334 DBD	-		-	3.3	8.9[1]	1.2[1]	-	-	-	-	85	77	-	88	78	-
Skaro *et al*[36]	Chicago, United States	2009	32 DCDc	15.8	5.5 h	-	3	53[1]	38[1]	93	-	-	2[1]	61[1]	53[1]	-	74	74	-
2003-2008			237 DBD	-	5.2 h	-	1	22[1]	2[1]	-	-	-	27[1]	85[1]	74[1]	-	90	81	-
Jay *et al*[107]	Chicago, United States	2010	28 DCDc	16.5	5.7 h	1.8 yr	3.6	57.7[1]	44[1]	10.7	7.1	-	21.4[1]	60[1]	50[1]	-	70[1]	70[1]	-
2004-2008			198 DBD	-	5.3 h	-	0.5	21[1]	1.6[1]	3	6.1	-	7.1[1]	89[1]	78[1]	-	96[1]	93[1]	-
Dezza *et al*[92]	Ghent, Belgium	2007	13 DCDc	10	6.16 h	163 d	8[1]	-	23.1[1]	-	-	-	31[1]	54[1]	-	-	62[1]	-	-
2003 -2006			98 DBD	-	9.14 h	603 d	1[1]	-	-	-	-	-	12[1]	79[1]	-	-	86[1]	-	-
Maheshwari *et al*[95]	Johns Hopkins Balti-		20 DCDc	33	8.7 h	-	5	60	50	5	-	-	20	62	62	30	78	78	40
1997-2006	more, United States																		
Muiesan *et al*[70]	London,	2005	31 DCDc	14.7	8.6 h	-	3.1	9.4	0	3.1	-	28.1	3.1	86.5	-	-	89.6	-	-
2001-2004	United Kingdom																		
Abou Abbass *et al*[97]	United States	2010	26 DCDc	39	5.3 h	-	0	46	15.4	11.5	7.7	26.9	23	77	-	-	92	-	-
2004-2008																			
Detry *et al*[94]		2010	58 DCDc	25	451	-	3.4	38	32.7	3.4	3.4	-	13.8	72.4	48.8	-	83.3	66.9	-
2003-2007																			
Hernandez-Alejandro *et al*[42]	London,	2010	10 DCDc	54.7	5.8 h	-	10	10	0	0	0	-	10	-	-	-	-	-	-
2006-2007	United Kingdom																		
Hashimoto *et al*[96]	United States	2010	22 DCDc	21	422	-	4.5	27	9	0	0	-	9	81	81	-	-	-	-
2005-2009																			

DCDc: Controlled donors after cardiac death; DBD: Donors after brain death; SCD: Standard criteria donors; ECD: Extended criteria donors; WIT: Warm ischemia time; CIT: Cold ischemia time; PNF: Primary non-function; ITBL: Ischemic-type biliary lesions; HAT: Early hepatic artery thrombosis; HAS: Early hepatic artery stenosis. Major symptomatic biliary complications include biliary leak, anastomotic and non-anastomotic stenosis. [1]Numbers denote the statistically significant difference between groups.

Biliary epithelium is also known to be sensitive to cold preservation-reperfusion injury and the correlation between the incidence of ITBL and the duration of cold ischemia has been well documented. Li *et al*[111] demonstrated that the rate of ITBL is significantly increased in livers with increased preservation injury, as reflected by post-transplant peaks in serum transaminases. Other variables implicating in the mechanisms of ITBL may include injury of the peri-biliary vascular plexus, bile salt toxicity and potential immunological etiologies (ABO incompatibility, liver diseases with autoimmune component like autoimmune hepatitis and primary sclerosing cholangitis)[102]. Chan *et al*[43] found donor age > 50 years, donor weight ≥ 100 kg and total ischemia time ≥ 9 h were predictive for the development of ITBL. Patients who underwent LT from DCD donors > 60 years had a markedly high rate of biliary complications (67%), with a RR of 5.6 (95% CI: 0.98-32.2)[34].

Due to serious consequences of ITBL on the patient's quality of life and healthcare cost, preventive measures seem to play a pivotal role in the safe expansion of DCD liver use. Attempts to minimize biliary duct damage may include the use of normothermic ECMO for donor maintenance[29,71,112] and machine perfusion for liver grafts, choice of preservation solutions [histidine-tryptophan-ketoglutarate (HTK) *vs* University of Wisconsin][113-117], use of anticoagulation and thrombolytic agents[96], extensive

Table 4 Results of donation after cardio-circulatory death-liver transplantations in United Network for Organ Sharing data base registry

Authors and study period	Publication year	Patient number and Maastricht category	WIT (min)	CIT (h)	PNF (%)	Retransplantation (%)	Graft survival %			Patient survival %		
							1 yr	3 yr	5 yr	1 yr	3 yr	5 yr
Abt *et al*[67]	2004	144 DCD	12.7	8.1	11.8[1]	13.9[1]	70.2[1]	63.3[1]	-	79.7	72.1	-
1993-2001		26 856 DBD		8.9	6.4[1]	8.3[1]	80.4[1]	72.1[1]		85	77.4	
Mateo *et al*[74]	2006	367 DCD	15.6	8.3	-	-	71[1]	60[1]	53[1]	-	-	-
1996-2003		33 111 DBD		8.4			80[1]	72[1]	65[1]			
Lee *et al*[32]	2006	874 DCD	15.4	7.9	-	-	72.1[1]	61.8[1]	38.8[1]	82.3[1]	75.9[1]	65.3[1]
1996-2006		43 734 DBD		8.2			80.7[1]	71.9[1]	65.6[1]	85.4[1]	77.5[1]	71.5[1]
Doshi *et al*[68]	2007	345 DCD	-	8.2	6.4[1]	13.0[1]	75	65	-	83	77	-
1998-2004		20 289 young-DBD		8.1	3.9[1]	5.6[1]	83[1]	75[1]		88[1]	80[1]	
		3604 old-DBD		8.2	5.3[1]		76	64		83	73	
Merion *et al*[125]	2006	472 DCD	-	7.9	-	-	70.1[1]	60.5[1]	-	-	-	-
2000-2004		23 598 DBD		8.1			83[1]	75[1]				
Selck *et al*[69]	2008	855 DCD	-	-	-	21.6[1]	73.8[1]	57.6[1]	-	-	-	-
2002-2007		21 089 DBD				8.8[1]	84.4[1]	74.4[1]				
Mathur *et al*[127]	2010	1567 DCD	16.1	7.5	-	13.6	-	-	-	78	64.9	-
2001-2009												

DCD: Donors after cardiac death; DBD: Donors after brain death; WIT: Warm ischemia time; CIT: Cold ischemia time; PNF: Primary non-function. Major symptomatic biliary complications include biliary leak, anastomotic and non-anastomotic stenosis. [1]Numbers denote the statistically significant difference between groups.

irrigation of the donor bile duct and pressure perfusion of the hepatic artery during organ retrieval and/or at back table[113,118,119], early porto-caval shunt to reduce portal hypertension in the recipient, choice of reperfusion techniques (concomitant *vs* sequential reperfusion of portal vein and hepatic artery)[120] and certainly the most important thing is always minimizing warm and cold ischemia period[121].

HAT and stenosis

HAT is a thrombo-embolic occlusion of the hepatic artery that can occur early or late after LT. Most authors used the first 30 d post-transplant as a time point to distinguish between early and late HAT[122]. Early HAT results in fulminant hepatic failure, bile duct necrosis and leaks, relapsing bacteremia and ultimately graft loss and recipient death. The frequencies of early HAT after DCD-LT varied from 0% to 16.6% and did not seem significantly higher than those after DBD-LT in most studies[20,29,34,36,43,51,71,72,89,91,104,105] except Yamamoto *et al*[90] (33.3% *vs* 0%). Risk factors for early HAT have been well analyzed in a recent systemic review[123]. Few detailed studies discussed late HAT.

The incidence of hepatic artery stenosis (HAS) was not consistently found higher in DCD than DBD grafts (12.8%-16.6% *vs* 0%-5.4%)[72,91]. It is possible that hepatic arteries are susceptible to WI during DCD organ retrieval, resulting in subsequent scar and stenosis. Moreover the increased susceptibility of DCD livers to post-operative arterial ischemia might be responsible for more biliary strictures in DCD than DBD recipients with HAS (83% *vs* 37%) as well as shorter time to the development of biliary strictures after HAS in the DCD group[91]. Inadequate surgical technique, vascular trauma by clamps, graft rejection, recurrent hepatic disease might also play a role in the mechanisms for HAS[72,124].

Graft and patient survival

Graft survival is defined as the time from transplantation to either re-transplantation or patient death, with "early" and "late" graft failure occurring within and beyond 1 year post-transplant, respectively[34]. Few studies reported experience with LT from uncontrolled DCD donors. Early results were poor with a PNF rate of 50% and one-year graft survival rate of only 17%[85] leading to a scarce usage of this donor category in the United States. Subsequent series in Spain using advanced *in vivo* organ preservation methods showed promising outcomes with one- and five-year graft survival rates of 50%-80% and 49%, and one- and five-year patient survival rates of 70%-85.5% and 62%, respectively[29,71,89]. LT from controlled DCD donors offered better results although they still appeared inferior to DBD-LT in matched studies[34,72], registry data analysis[32,67-69,74,125] and in some comparative studies[36,91,92]. One-, three-, five- and ten-year graft survival rates were 54%-79.5%, 53%-74.5%, 37.5%-71% and 37.5%-44%, respectively. Patient survival rates at corresponding time points were 61.9%-91.5%, 62.8%-89.5%, 42.9%-89.5% and 42.9%-57%, respectively. Transplant outcomes comparable to those obtained from DBD-LT have been sporadically reported in select centers through careful donor selection and optimization of CIT or through invasive techniques designed to optimize recovery before declaration of death[20,43,51,104].

Significant risk factors for DCD liver graft loss have been identified by multivariate Cox regression technique in both single center studies and large data registry analysis[32,67-69,74,126,127]. Among donor risk factors, age > 50 years, total WIT > 30-35 min, CIT > 6 h, body weight > 100 kg and regional or national liver distribution had deleterious effects on graft survival[32,74,127]. There is a stepwise increase in the relative risk of graft failure among donor age, WIT and CIT[32,127]. Strong recipient determinants of

graft failure include age > 55 years, history of previous transplantation, medical status at transplantation [intensive care unit (ICU) or non-ICU hospitalization, life support, dialysis, renal insufficiency], high MELD score (> 30) and positive HCV serology[32,74,127]. In the DBD-LT model, it has been shown that a single risk factor lessened outcome marginally, however, the additive effect of multiple risk factors in a given donor-recipient pair were disastrous[83]. Grafts with ≥ 3 donor risk factors had significantly lower 1-year post-transplant survival than no or only 1 or 2 risk factors (58.3% *vs* 72.6%, 69.2% and 73.9%, respectively). No grafts with 4 risk factors survived within 1 year[128]. The relative risk of allograft failure from LT utilizing DCD donors was 31%-87% higher than LT utilizing DBD donors[67-69,125,126]. Causes of early graft failure included PNF, biliary complications, HAT and deaths from sepsis/multi-organ failure. Late graft failure was often secondary to chronic rejection and recipient death with a functioning graft.

Although DCD livers may not be as good as DBD ones with potential inferior transplant outcomes, there are subgroups of grafts and recipients that could give favorable results through appropriate graft and recipient matching. Low-risk DCD grafts which are transplanted in low-risk patients lead to comparable graft survival rates with DBD livers. Livers from DCD donors transplanted into high-risk recipients fared poorly independent of the allograft quality[74]. Doshi *et al*[68] showed DCD liver grafts were not inferior to DBD livers from older donors (≥ 60 years). Given the ever increasing demand for LT, DCD livers appear to be a reasonable alternative to increasing use of older or split livers and are a reasonable option when death is imminent[68]. Even if graft or/and patient survival is lower with a DCD liver, it is still better than dying because of turning down a DCD offer and continuing to wait for a DBD liver on these d as the patient' s choice is frequently not between marginal livers (including DCD) and standard livers but between marginal livers and no livers[105]. The benefit of earlier access to LT provided by a DCD graft could outweigh the risks of prolonged waiting for a standard graft[77].

Re-transplantation

DCD recipients more often require re-transplantation. Respectively, 21.6%-42% *vs* 8.8%-16% of DCD and DBD recipients were listed for re-transplantation[36,69]. The re-transplantation rate ranged from 7.6% to 31% in DCD-LT compared to 2.5%-12% in DBD-LT[20,29,34,36,51,67-69,71,72,91,92,106]. DCD livers exhibited a 2.1 times greater risk of graft failure, a 2.5 times greater risk of re-listing, and a 3.2 times greater risk of re-transplantation compared with DBD livers[36]. The majority of re-listing and re-transplantation in the DCD group were a consequence of biliary complications, especially ischemic cholangiography, but not due to an increased incidence of PNF, HAT or technical complications[36,69]. Particularly DCD livers had a temporally different failure pattern within the first year post-transplant that limited access to re-transplantation[36,69]: graft failure was more likely to occur within the first 180 d (18.1% *vs* 11.7%[67], 10.2% *vs* 2.5%[72] and 20.5% *vs* 11.5%[69] of DCD and DBD grafts failed within 60, 90 and 180 d, respectively); at re-transplantation, DCD recipients waited longer and received higher risk allografts; and more DCD recipients remained waiting for re-transplantation with fewer removed for death, clinical deterioration, or improvement. Re-transplantation arouses controversy on medical, economic, and ethical grounds: patient and graft survival rates after a second LT are inferior to those after initial grafting, the procedure is more expensive and in the context of organ shortage, re-transplantation inevitably denies organs to first-time recipients[129].

Utilization of DCD allografts for re-transplantation was rare (2.5% of initial DCD *vs* 3.1% of initial DBD) and outcomes from each group were comparable[69]. The general practice is to avoid re-transplantation with a DCD graft[36]. The use of DCD donors in the setting of re-transplantation resulted in an increased risk of recipient death (hazard ratio = 2.1, 95% CI: 1.2-3.6)[129].

Acute rejection

The acute rejection rate did not differ significantly between DCD- and DBD-LT in most studies (1.9%-29% *vs* 0.6%-34%)[20,72,87,89,104]. Foley *et al*[91] reported a one-year rejection rate of 61% in the DCD group similar to that in the DBD group (56%). There were little data looking at the impact of DCD source on the risk of acute rejection.

EXPERIMENTAL STRATEGIES TO IMPROVE DCD-LT OUTCOMES

The progressively increased DCD liver procurement to solve the shortage of DBD organs and to alleviate the waiting-list mortality has raised many challenges to the transplant community and transplant policy makers[110]. A lot of experimental researches have been performed over the past decade, intervening in both donors and recipients at different phases of the transplantation process, at the aim of tackling some of these challenges and providing a deep insight into IRI mechanisms.

Donor pre-treatment

Various cyto-protective substances have been successfully administered into the donor prior to cardiac arrest for prevention of liver microcirculatory disturbance. Microcirculatory disturbance was the main obstacle to successful DCD-LT, which was due to four major mechanisms: deterioration of sinusoidal endothelial cells (SEC) caused by activated Kupffer cells, sinusoidal narrowing caused by some vasoconstrictors and swollen hepatocytes, leukocyte and platelet adhesion, and hyper-coagulability[130]. Up to now, only *Heparin* and *phentolamin* (an anticoagulative substance and alpha-adrenergic antagonist) are allowed in clinical DCD organ procurement[131], other substances remain in animal models. *Tacrolimus,* besides

its powerful immunosuppression, enabled to prevent liver normothermic IRI by multiple mechanisms[132]. *Milrinone*, a type 3 phosphodiesterase inhibitor, attenuated graft injury caused by warm and cold ischemia *via* an increase in intracellular cAMP levels, protection of SEC, relaxation of hepatic stellate cells, inhibition of platelet aggregation and anti-inflammatory effect[133]. *Lazaroids*, an antioxidant designed to inhibit iron-dependent lipid peroxidation, ameliorated SEC viability *via* antioxidant effects and membrane stabilization[134]. *N-acetylcystein* has a direct effect on oxygen free radicals, but its usage had no effect in both graft viability and lipid peroxidation[135].

Animal studies clearly showed the concept of pharmacological modulation of organ donors before procurement is feasible to improve the viability of marginal grafts. Nevertheless there are no definitive recommendations for the use of these drugs. Application of this method to clinical LT would require management of some practical problems and possible ethical conflicts[136].

Organ preservation

Preservation of DCD livers by hypothermic machine perfusion (HMP) was shown superior to static cold storage (SCS) in many experimental studies[137,138].

Nonetheless a putative drawback of HMP for livers is to induce alterations at the vascular endothelial site, especially if HMP was performed for a long time or under suboptimal conditions[139]. Endoplasmic stress activation promoted cellular apoptosis *via* activation of caspase-12[140,141]. The efficiency of HMP was markedly increased by oxygenation of the perfusate[142]. The concern that high oxygenation might favor the generation of oxygen free radicals, which in turn could impair tissue integrity, was not justified. Several investigators could demonstrate the beneficial effect of oxygenated HMP in reducing the liver expression of pro-inflammatory cytokines (TNF-α, IL-8), adhesion molecules (ICAM-1) and major histocompatibility complex class Ⅱ antigens[143-145]. This benefit will likely be more pronounced in marginal grafts such as elderly, steatotic and DCD livers[144]. Cyto-protective agents can be added into the machine perfusion (MP) solution to ameliorate the efficiency of HMP organ preservation[146].

The positive effects of HMP on warm-ischemically pre-damaged livers were observed even after a brief period of MP, before (pre-conditioning) or after SCS (post-conditioning)[143,147] and therefore it was not necessary to require MP over a full preservation period and helped avoid side-effects of HMP on vascular endothelium[141]. The use of HMP as the initial method for organ preservation followed by secondary SCS during transportation combined the advantage of aerobic resuscitation (i.e., restitution of cellular homeostasis) with an ease of SCS for later surveillance and transportation[141]. Manekeller *et al*[148] showed a post-conditioning of 1 h after SCS can ameliorate the viability of marginal livers. The extension or abbreviation of post-conditioning time seems to have no further beneficial effects[148].

Schön *et al*[66] reported advantages of normothermic machine perfusion (NMP) over SCS in pig DCD-LT models. Livers subjected to 1 h of WI and then cold-stored for 4-24 h were rendered completely nonviable while such livers under 4-24 h of oxygenated NMP recovered function to a viable level[149]. Due to the complexity of the logistics of clinical multi-organ recovery and of the NMP device, a period of cold preservation prior to warm perfusion of the liver is unescapable. A brief period of cold preservation (1 h) prior to NMP could maintain the synthetic and metabolic function but resulted in significant hepatocellular damage, sinusoidal endothelial cell dysfunction and Kupffer cell injury[150]. Once this duration was prolonged to 4 h, NMP completely failed to resuscitate porcine livers[151]. Normothermically perfusing DCD livers throughout the preservation period not only replenished cellular substrate, ameliorating the ischemic injury, but also provided a clear assessment of liver function and therefore could permit the use of severely injured organs with reassurance of function[149,152].

Despite the aforementioned benefits of MP over SCS in liver preservation, only SCS is clinically approved up to now, MP is still in the pre-clinical stage and early clinical studies[153]. Tojimbara *et al*[154] showed the impact of viscosity and temperature of initial flushing solutions on graft function. A low viscosity flushing solution was associated with lower vascular resistance, whereas a warm flush solution prevented cold-induced vasospasm and therefore improved the washout effect of the microcirculation[154]. HTK solution possessing a low viscosity and low potassium is more preferable in the DCD setting. The role of aeration of the cold-stored liver was also clarified. Oxygen provided either by surface diffusion (surface oxygenation) or intravascular diffusion (oxygen persufflation) helps improve the energy status of organs thus leading to earlier recovery. Surface oxygenation was not in use any more due to complicated technique, limited efficiency and risk of oxygen intoxication[155]. Venous systemic oxygen persufflation (VSOP) was shown to improve organ viability during hypothermic storage of the grafts and to be a feasible means for reconditioning of warm-ischemically pre-injured livers from DCD donors[155-158]. Experimentally even a short period of VSOP prior to long-term preservation of the liver by SCS may be sufficient for a relevant improvement of liver integrity upon reperfusion[159]. Gaseous persufflation with carbon monoxide was also tested in a DCD-LT rat model with enhanced liver graft viability[160]. However no additive or synergistic effect was noted when livers were persufflated with a mixture of gaseous oxygen and carbon monoxide[161].

Pharmaceutical interventions during SCS aimed at conditioning marginal organs also increasingly gained attention. Different cyto-protective drugs have been added into the flush and/or preservation solution, like vasodilators (phentolamin, epoprosterol, dopamine)[162,163], anti-coagulants (heparin), fibrinolytic agents (strepto-

kinase)[164], antioxydants (superoxide dismutase, edaravone)[165,166], antibiotics, hormones (glucagon, growth factors)[167]. In the DCD setting, vasodilators, anti-coagulants, thrombolytic agents and antibiotics seem particularly necessary because the organs tend to develop vasospasm, thrombus formation in the microcirculation and the risk of colonic bacterial contamination secondary to translocation of organisms during the WI period[168,169].

Viability testing

Due to serious consequences of transplanting a DCD liver with potentially severe IRI (PNF, re-transplantation or even recipient death), it would be ideal if the viability of such livers could be predicted prior to rather than after transplantation. WIT is not always exactly known and thus cannot be a reliable parameter. Light microscopic examination of biopsy specimens was unable to uniformly predict liver function after transplantation[170]. Monbaliu *et al*[171] showed the extent of parenchyma vacuolation predicted pig liver graft viability before LT. Muiesan *et al*[70] applied the mechanical digestion of liver biopsies with collagenase and assessed the viability of hepatocytes by trypan blue exclusion method. However, the test was not helpful and the decision as to whether to use the liver was generally made on gross appearance, ease of perfusion, degree of steatosis and donor characteristics[172].

Another approach is to evaluate the vascular resistance and enzyme release in the perfusate of HMP livers. Resistance index of the portal vein and hepatic artery showed no utility[173]. Biomarkers of liver cell damage, like transaminases, lactate dehydrogenase and liver fatty acid binding protein, correlated well with WI duration and concomitant hepatocyte damage in pig DCD-LT models[174]. Possible other parameters are the ATP content and redox active iron status of the liver during HMP[175]. During NMP, the assessment of liver viability may be easier because the liver is in a normal metabolic state. Bile production was a good viability indicator besides the measurement of other liver functions (detoxification, metabolism or synthesis)[176]. Recently Liu *et al*[177] has tested the utility of magnetic resonance imaging and proton magnetic resonance spectroscopy to evaluate WI livers without success.

Recipient treatment

Pharmaceutical strategies aimed at modulating IRI mechanisms were also applied successfully in animal recipients and generally did not impose ethical problems as donor pre-treatment. Such protocols without donor pretreatment will be favorable in clinical application. Most studies tested a single agent for a specific target of the IRI process. A multi-factorial approach acting on different pathways of the IRI process have been advocated and remarkably ameliorated transplant outcomes[162].

PERSPECTIVES

The future of DCD-LT is promising. Concerted efforts should concentrate on the identification of suitable donors (probably Maastricht category Ⅲ DCD donors), better donor and recipient matching (high risk donors to low risk recipients), use of advanced organ preservation techniques (oxygenated HMP and NMP, VSOP), and pharmacological modulation (probably a multi-factorial biologic modulation strategy) so that liver procurement and transplantation from DCD donors could be widely expanded and attain equivalent results as DBD-LT.

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REVIEW

Surveillance for gastrointestinal malignancies

Ashish K Tiwari, Heather S Laird-Fick, Ramesh K Wali, Hemant K Roy

Ashish K Tiwari, Heather S Laird-Fick, Department of Internal Medicine, Michigan State University, East Lansing, MI 48824, United States
Ramesh K Wali, Hemant K Roy, Department of Internal Medicine, North Shore University Health System, Evanston, IL 60021, United States
Author contributions: Tiwari AK, Laird-Fick HS, Wali RK and Roy HK contributed equally to this paper.
Correspondence to: Ashish K Tiwari, MD, Department of Internal Medicine, Michigan State University, 138 Service Rd No. A225, East Lansing, MI 48824,
United States. ashish.tiwari@hc.msu.edu
Telephone: +1-312-2088961 Fax: +1-517-4322759
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Abstract

Gastrointestinal (GI) malignancies are notorious for frequently progressing to advanced stages even in the absence of serious symptoms, thus leading to delayed diagnoses and dismal prognoses. Secondary prevention of GI malignancies through early detection and treatment of cancer-precursor/premalignant lesions, therefore, is recognized as an effective cancer prevention strategy. In order to efficiently detect these lesions, systemic application of screening tests (surveillance) is needed. However, most of the currently used non-invasive screening tests for GI malignancies (for example, serum markers such as alpha-fetoprotein for hepatocellular carcinoma, and fecal occult blood test, for colon cancer) are only modestly effective necessitating the use of highly invasive endoscopy-based procedures, such as esophagogastroduodenoscopy and colonoscopy for screening purposes. Even for hepatocellular carcinoma where non-invasive imaging (ultrasonography) has become a standard screening tool, the need for repeated liver biopsies of suspicious liver nodules for histopathological confirmation can't be avoided. The invasive nature and high-cost associated with these screening tools hinders implementation of GI cancer screening programs. Moreover, only a small fraction of general population is truly predisposed to developing GI malignancies, and indeed needs surveillance. To spare the average-risk individuals from superfluous invasive procedures and achieve an economically viable model of cancer prevention, it's important to identify cohorts in general population that are at substantially high risk of developing GI malignancies (risk-stratification), and select suitable screening tests for surveillance in these cohorts. We herein provide a brief overview of such high-risk cohorts for different GI malignancies, and the screening strategies that have commonly been employed for surveillance purpose in them.

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Key words: Gastrointestinal malignancies; Surveillance; Screening; Biomarkers; Cancer prevention

Peer reviewer: Dr. Ki Baik Hahm, Professor, Department of Gastroenterology, Gachon Graduate School of Medicine, Lee Gil Ya Cancer and Diabetes Institute, 7-45 Songdo-dong, Yeonsu-gu, Incheon 406-840, South Korea

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INTRODUCTION

Malignancies originating in the gastrointestinal (GI) tract are responsible for about one third of global cancer burden[1]. In the United States, of estimated 1 529 560 new cancer cases and 569 490 cancer deaths in 2010, approximately 274 330 new cases (approximately 18% of total) and 139 580 deaths (approximately 25% of total) could be attributed to GI malignancies[2]. One of the reasons behind relatively high mortality rate for GI cancers is their visceral location, which requires highly invasive endoscopy to directly visualize early-stage lesions, leading to diagnoses at advanced incurable stages in most cases.

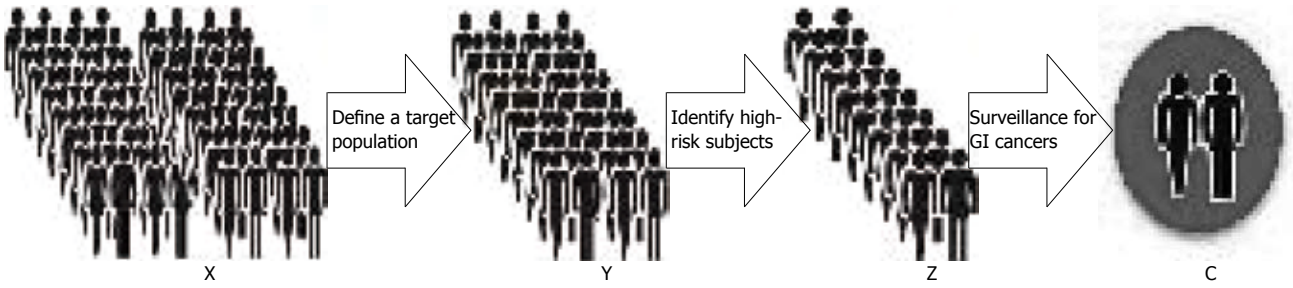


Figure 1 Risk stratification is a step-wise approach towards identifying high-risk individuals where surveillance for gastrointestinal cancers is truly needed in order to detect any neoplastic growth at early stages. The initial step is to define a target population (Y) in the general population (X) where screening strategies would be applied. Based upon findings of the screening tests in target population, subjects deemed to be at high-risk for developing a gastrointestinal (GI) malignancy (Z) would need repeated screening tests at suitable intervals (surveillance), and this would result in detection of potentially curable early stage cancerous lesions in certain individuals (C).

Secondary prevention through systemic application of screening tests (surveillance) to detect cancer-precursor/premalignant lesions is regarded an effective prevention strategy, and arguably also promotes positive life-style changes[3]. However, only a small proportion of total population is indeed at a credible risk of developing GI malignancy in future and needs surveillance. Identifying these high-risk cohorts (risk-stratification) is therefore crucial for the success of any surveillance program[4] (Figure 1). Such an approach is not only mandatory from an economic perspective but also spares the general population of repeated invasive screening tests to detect cancer precursor lesions. Screening tests detect the end-point of increased genetic/environmental predisposition (that is precursor lesions), and therefore constitute the most important arm of any risk-stratification strategy.

CANCER BIOMARKERS, SURROGATE MARKERS, LEAD-TIME BIAS AND OVERDIAGNOSIS BIAS

Biomarker is a variable that directly relates to cancer progression and/or final biological outcome (such as death), and is measured by a screening test[5]. The ease of procuring the material for biomarker analysis is an important question for visceral organs such as those in GI tract. Intraepithelial neoplasia or dysplasia remains the most reliable marker of impending malignancy, but is associated with a number of inherent limitations, including the need for invasive procedures to obtain tissue and inter-pathologist variability in interpretation of histopathological features[6]. Moreover, although endoscopic techniques have evolved over the years[7], their regular use for risk-stratification in average risk populations is economically unviable at this point. Surrogate markers, which variably correlate with cancer progression, can usually be measured through non-invasive means such as in serum or stool and therefore represent attractive tools for screening, but have limitations such as poor sensitivity and/or specificity[8]. It should be noted that surveillance programs are recommended only if an effective treatment is known to exist should any precursor/premalignant lesion be detected upon screening. This is important in order to avoid the possibility of lead-time bias (early diagnosis of cancerous lesions leading to increased number of years patient survives without actual shift in age at death) and overdiagnosis bias (diagnosis of cancerous lesions that won't have progressed, meaning they won't have caused death in first place)[9].

ESOPHAGEAL CANCER

In the United States, more than four fifth of patients diagnosed with esophageal cancer in 2010 are estimated to have a cancer death[2]. Esophageal adenocarcinoma (EA) and squamous cell carcinoma (ESCC) together account for more than 95% of esophageal cancer cases: ESCC being responsible for the bulk of cases worldwide and EA being more common in western countries, particularly among Caucasian males[10]. Due to such stark differences in the epidemiology of ESCC and EA, there is greater emphasis on risk-stratification for ESCC in developing countries, as compared to EA and its precursor lesion, Barrett's esophagus (BE), in developed countries. However, with increasing westernization of developing countries, the incidence of EA is surely on the rise in these countries too.

EA

Nearly all the cases of EA evolve through BE→Dysplasia→EA sequence[11]; and therefore, BE subjects represent a high-risk cohort where surveillance could be considered depending upon mucosal changes detected on endoscopic biopsy (reviewed in detail in reference[12]). The challenging part, however, is to identify individuals who could have BE in first place. Long-standing gastroesophageal reflux disease (GERD) patients are clearly at-risk for developing BE, but a significant proportion of BE cases can exist and progress to EA even without GERD symptoms. Furthermore, high prevalence of GERD symptoms in general populations (> 20% of adult population[13]) and extremely low rate of progression from GERD to BE to EA (approximately 0.5% and 1.0% per year respectively[14,15]) means that endoscopic screening for BE in all GERD patients would not be cost-effective[16]. However, certain subsets of GERD patients can clearly benefit from screening for BE on

an individual basis; for example, first-degree relatives of BE patients are more likely to harbor BE in presence of GERD symptoms as compared to other GERD patients with no such family history[17,18]. This increased risk could be related to genetic polymorphism of cyclin D1 and glutathione S-transferase genes which have been implicated in development of BE[19-21]. However, it's important to emphasize that there is no evidence of clear benefit of screening in asymptomatic persons (no GERD symptoms) with a positive family history of BE/EA. Overall, screening recommendations for BE remain controversial, and > 95% of EA cases are still diagnosed in patients without any prior diagnosis of BE[22]. The risk of EA (as well as ESCC) is also increased in hereditary conditions such as Peutz-Jeghers syndrome (PJS), but endoscopic surveillance in such cases is recommended for the whole upper GI tract and is not esophagus-specific.

A recent single cohort study demonstrated promising results of non-endoscopic screening for BE using an ingestible esophageal sampling device (Cytosponge) coupled with immunocytochemistry for trefoil factor 3[23]. However, non-endoscopic screening is still tested only on a limited scale, and serum markers have not been shown to be effective for screening for BE/EA as yet. Therefore, standard endoscopy with biopsy remains the "gold" standard for detecting BE. However, apart from the need of invasive endoscopy, low positive predictive value (about 34%) associated with index endoscopy[24] and need for multiple biopsies (at least 8 biopsies[25]) to diagnose metaplasia needed to define BE have been major drawbacks of endoscopy based screening. Recent advancements in endoscopic GI mucosa imaging (reviewed in reference[26]) have largely improved lesion detection capabilities, and enabled targeted biopsy of the dysplastic areas. Current recommendations for the need and frequency for EA surveillance in BE patients are largely based upon the degree of dysplasia in the BE mucosa (reviewed in detail by Badreddine *et al*[12]). In summary, after screening endoscopy in suspected BE patients (such as GERD patients over 50), detection of no dysplasia leads to repeat confirmatory endoscopy after 6-12 mo followed by endoscopic surveillance every 3 years; detection of low grade dysplasia leads to repeat confirmatory endoscopy in 6 mo followed by yearly endoscopic surveillance; and detection of high grade dysplasia needs confirmation by two expert pathologists and either 3 monthly surveillance combined with multiple biopsies spaced at every 1 cm *vs* endoscopic ablation *vs* esophageal resection.

ESCC

Currently, surveillance for ESCC is mandated only in two conditions-Tylosis palmaris (an obscure skin condition often associated with internal malignancies) and Lye ingestion[27]. However, co-existence of multiple ESCC risk factors could prompt surveillance in certain circumstances. For example, alcohol, smoking, flushing response to alcohol, Asian ethnicity, inactivating aldehyde dehydrogenase 2 allele polymorphism and personal history of any other malignancy of aerodigestive tract (UADT-which includes oral cavity, larynx, pharynx and esophagus) are all independent risk factors for ESCC[28-31]; and although none of them warrants surveillance for ESCC on its own, it would be worthwhile to consider screening for precursor lesions in persons with multiple risk factors, especially in case of Asian ethnicity, on an individualized basis. Some other conditions such as achalasia and Plummer-Vinson syndrome that are known to increase the risk of ESCC warrant endoscopic interventions for symptomatic treatment (such as for dysphagia) but not for screening[32]. Most of the reports on the impact of screening on ESCC incidence and mortality, and associated cost-effectiveness analyses have come from geographically high-risk countries. In two such studies conducted in China, investigators concluded that screening general population with exfoliative balloon cytology (EBC) was an effective tool for risk stratification and could have favorable impact on ESCC incidence and mortality[33,34]. However, United States-based Veterans' Affairs (VA) studies conducted in relatively high-risk population due to personal history and (or) symptoms produced conflicting results; and it was concluded that because of the low prevalence of ESCC in the United States and the difficulty of diagnosing malignancy in the setting of active esophagitis, EBC was probably not a cost-effective screening strategy in Western world[35]. Therefore, internationally, endoscopy aided biopsy therefore remains the standard test for ESCC screening and surveillance currently, albeit only in a limited cohort of subjects at high-risk for ESCC.

GASTRIC CANCER

Gastric cancer (GC) is the second leading cause of cancer deaths worldwide, and remains a major public health burden in Asia-pacific countries such as China, Japan and Korea where the age-standardized incidence rate for GC is > 20 per 100 000 subjects (defining criterion for high-risk areas)[1]. Gastric adenocarcinoma is the most common gastric malignancy (> 90% cases), with two subtypes: Intestinal (more common form and prevalent in high-risk areas) and diffuse type. Due to stark geographical differences in the prevalence of gastric cancer worldwide, the strategies and significance attached with screening for this cancer are highly variable.

The individuals migrating from high-risk areas remain at-risk even in low-risk countries such as the United States; however, their offspring tend to have risk levels comparable to that of the local population[36]. Geographical origin and location is therefore an extremely important consideration for any surveillance strategy against GC. Universal screening for GC has been considered only in certain high-risk countries such as Japan, South Korea and Matsu Island in Taiwan (China)[37]. On the other hand in average/low-risk countries, screening is recommended only in the presence of a well-characterized familial predisposition to GC (responsible for 1%-3% of GC cases), such as in case of hereditary

diffuse gastric cancer (HDGC) syndrome, familial adenomatous polyposis (FAP), PJS and Lynch syndrome. HDGC is the most common inherited form arising due to germline mutation in *E-cadherin* gene (*CDH1*) with the carrier of the mutations having more than 80% lifetime risk of developing GC[38]. However, surveillance or genetic testing is not considered for poorly-characterized familial cases (responsible for 8%-10% of GC cases) which are believed to be associated with more common but less penetrant defects such as polymorphism in pro-inflammatory interleukin-1 (*IL-1*) gene clusters and toll-like receptors 4 (TLR 4) + 896A > G[37,39,40]. Additionally, TLR 4 + 896A > G polymorphisms in TLR 4, a pattern recognition receptor that activates pro-inflammatory signaling pathways in response to microbes, has been associated with presence of GC and its precursors which indicates the relevance of TLR 4 polymorphism during gastric carcinogenesis[41]. A meta analysis of the role of IL-1b and IL-1 receptor antagonist gene polymorphisms in gastric cancer risk showed an association in Caucasians, but not in Asians[40]. Similarly, a metaanalysis by Huang *et al*[42] concluded that *cag A* seropositivity significantly increased the risk for gastric cancer and could be used for identifying populations at risk for GC. However, despite high prevalence of *cagA* in Asia-Pacific regions, the currently known *cagA* genotypes in Asia are not associated with increased GC risk[43]. Other high-risk subgroups considered for screening on a case-to-case basis are elderly patients with atrophic gastritis or pernicious anemia, patients with partial gastrectomy, patients with the diagnosis of sporadic adenomas, and immigrant ethnic populations from GC high-risk countries.

Over ninety percent of GC cases are sporadic, and most are linked to *Helicobacter pylori* (*H. pylori*) infection[44,45]. A meta-analysis of six major studies on *H. pylori* eradication demonstrated that *H. pylori* "screen-and-treat" strategy reduced the incidence of GC[46]. Based on this, the Asia-Pacific Gastric Cancer Consensus Conference in 2008 concluded that it might perhaps be the right time for a population-based screening and treatment of *H. pylori* infection (by using locally approved screening tests for *H. pylori,* such as serum or stool antibody/antigen detection), particularly in high-risk areas as a part of GC prevention program[37]. Interestingly, Ford *et al*[47] have proposed that even in western countries where better sanitation, low-salt intake and effective treatment of *H. pylori* infection has led to gradual decline in GC incidence over decades, a "screen and treat" strategy for *H. pylori* could reduce the dyspepsia-related health care costs over a longer (10 years or more) follow-up duration. However, prospective trials on a global scale are needed to validate such observations; and currently, no screening for *H. pylori* is recommended for asymptomatic individuals in geographically low/average-risk areas. Gastric cancer phenotype initiated due to *H. pylori* is characterized structurally by a corpus predominant gastritis, multifocal gastric atrophy, intestinal metaplasia, and physiologically by high gastrin, low acid secretion, low pepsinogen I and pepsinogen I / Ⅱ ratio, and hypo-and achlorhydria[48-50]. All these findings have been used to design screening tests for GC, such as serum pepsinogen I levels and pepsinogen I / Ⅱ ratios that have been investigated in high-risk areas, but have limited usefulness on a global scale[51]. Currently, endoscopy aided with advanced imaging techniques and biopsy (at least 5) to look for precursor lesions remains the main tools for screening and surveillance for GC.

PANCREATIC CANCER

Pancreatic cancer (PC) is the most aggressive GI malignancy that silently progresses to untreatable metastatic disease in most cases, and is generally fatal within six mo of diagnosis[2]. However, interestingly, a study from Japan has demonstrated that resection of all pancreatic lesions < 1 cm in size can achieve about 100% cure rates[52], suggesting that a thorough surveillance program for PC could potentially be useful. However, this approach leads to unnecessary high-risk surgical resection of many benign lesions that won't have progressed to malignancy in first place. Therefore, identifying high-risk cohorts where such pancreatic lesions are more likely to be malignant is definitely a better-refined strategy for prevention. Such high-risk cohorts for PC (defined as having > 10-fold increased risk of PC as compared to the general population) include familial and/or syndromic cases (3%-16% of total cases) where screening is routinely recommended (Figure 1)[53-56]. However, screening strategy for individuals at 5- to 10-fold increased risk of pancreatic cancer (e.g., those with just one or two affected first-degree relatives) is unclear. Clearly, in such cases, most centers take individualized approaches depending upon the cost and other considerations. Future studies are needed to establish the risk threshold at which screening is likely to be most cost-effective.

Notably, conditions such as chronic pancreatitis, diabetes mellitus and smoking history have strong associations with PC, but none of them increases the risk to an extent that could warrant screening.

Screening for PC faces a unique challenge in terms of incidental radiological findings in the pancreas due to rampant use of computerised tomography (CT) scan in patient-care. Because many of these lesions are non-lethal, it's important to establish their malignancy potential in order to guide their management and avoid over-enthusiastic and sometimes unwarranted surgeries that could ensue otherwise[57]. The most common of these lesions are intraductal papillary mucinous neoplasms (IPMNs). IPMNs which involve the main duct have a 70% risk of containing a malignancy at the time of diagnosis and need to be resected[58], while those involving the branch ducts have 25% risk of containing malignancy and 15% risk of malignant transformation during follow-up, and they can be safely observed with continued surveillance[57,58]. Certain other features however necessitate immediate resection, such as diameter ⩾ 3 cm, a mural nodule appearance, main pancreatic duct dilation ⩾ 6 mm, progressively changing lesion characteristics,

or presence of symptoms[57,58]. In general, the approach is usually much more aggressive if such lesions are present in high-risk individuals[55].

Another variety of PC precursor lesions, although not detectable by routine imaging tests in a clinical setting, are pancreatic intra-epithelial neoplasia (PanIN)[59]. PanIN is a histological diagnosis where pro-cancerous genetic and epigenetic aberrations have been noticed. PanIN-3 lesions are essentially treated as PC and resected whereas PanIN-1 lesions have very small risk of malignancy, and can be safely followed-up[60]. The management of PanIN-2 lesions is controversial and recommendations depend upon co-existing conditions and cost-considerations.

Currently, endoscopic ultrasonography (EUS) is most efficient screening test for PC; it accurately identifies pancreatic cysts and IPMNs, and has the advantage of detecting structural changes somehow predictive of PanIN lesions[61]. Other screening modalities like CT and endoscopic retrograde cholangiopancreatography have fallen out of favor mainly due to low sensitivity and radiation exposure and high incidence of pancreatitis respectively. However, magnetic resonance imaging/magnetic resonance cholangiopancreatography (MRCP), especially secretin-enhanced MRCP, is still an acceptable alternative to EUS[62]. In the absence of any clear guidelines for the frequency and starting age for screening for pancreatic cancer, recommendations are highly institutionalized based upon factors such K-ras mutations, family history *etc*.[63-66].

HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy, and is increasing in incidence in the United States[2]. In general, 5-year survival is less than 10% if diagnosed in symptomatic patients, but HCC diagnosis prior to appearance of symptoms offers the curative opportunities through resection of tumor[67]. Although extremely rare in asymptomatic populations, HCC is a dreaded long-term complication of most chronic liver diseases (CLD); and therefore, CLD patients constitute the obvious target population for risk-stratification. However, the level of HCC risk varies in different CLDs; and due to the generally protracted course of CLDs and high cost of repeated screening, cost-considerations are very important in formulating surveillance strategies in these patients. Among CLD patients, diagnosis of cirrhosis is a strong predictor of the risk of progression to HCC, and advent of cirrhosis often marks the starting point for surveillance recommendations in these patients. Chronic viral hepatitis (hepatitis B and C) is the most common etiology behind HCC. Hepatitis B and C carriers have an HCC incidence rate of 0.2%-0.6% per year, and 3%-11% per year respectively[68,69]. Hepatitis B can particularly be deceptive because HCC can occur even in non-cirrhotic hepatitis B virus (HBV) carriers, and the risk seems to be variable. Asian ethnicity, viral replication status, and seropositivity for hepatitis B surface antigen and anti-HBe antigen differentially influence the risk of development of HCC in HBV carriers, and therefore have an important impact on surveillance program (reviewed in references[70-73]). On the other hand, the risk of HCC in long-term hepatitis C carriers is independent of factors such as ethnicity and viral replication status[71,74], and largely depends upon the extent and severity of cirrhosis. Based upon this, the first European association for study of the liver conference on HCC recommended screening for HCC in chronic hepatitis C patients with at least stage 3 fibrosis (METAVIR)[75]. Another puzzling question is whether surveillance for HCC should continue in successfully treated chronic viral hepatitis patients? Evidence has been conflicting for hepatitis B carriers with mostly Western and some Asian studies suggesting a significant reduction in HCC risk after successful treatment[76-78], and a non-randomized, but match controlled Asian study following a large cohort for longer periods suggesting continued risk of HCC post treatment[79]. Thus, given the higher risk associated with Asian ethnicity, it seems prudent to continue HCC surveillance in Asian hepatitis B carriers with cirrhosis even after successful seroconversion. However, the same can't be said about non-cirrhotic western populations. In contrast, continued surveillance for HCC is recommended for hepatitis C infected population because even after successful treatment (i.e., sustained virological response), the risk of HCC in cirrhotic hepatitis C carriers remains sufficiently high to warrant surveillance[74]. Additionally, co-existing risk factors like old age, viral genotype, viral replication status, aflatoxin exposure, co-infection and other CLDs, diabetes and human immunodeficiency virus also need to be taken into account in deciding the surveillance protocol for HCC in viral hepatitis carriers. Additionally, there are some non-viral cirrhotic conditions as well where surveillance could be considered (Table 1).

For screening purposes, ultrasonography (USG) and serum alpha-fetoprotein levels are often used. Serial USG (at 6-12 mo interval[80,81]) has by far been superior to any other screening test for HCC (65%-80% sensitivity and 90% specificity), and can detect nodules of 1 cm size, which are essentially curable[82]. However, USG needs to be aided with biopsies to differentiate between benign cirrhotic and dysplastic/malignant nodules. Conversely, evidence suggests that serum alpha-fetoprotein measurement has no role in HCC screening (although it can still have some utility in HCC diagnosis and follow-up), and should be no longer used for screening purposes. Other serological markers such as alpha flucosidase, glypican-3 and desgamma carboxyprothombin have already been discredited[83].

COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common cancer in both men and women[2]. Over the years, its incidence has constantly been decreasing, largely due to colonoscopic screening. As less than one third of CRC cases are as-

Table 1 At-risk cohorts for considering surveillance for gastrointestinal malignancies

Esophageal cancer
Barrett's esophagus
Tylosis palmaris
Lye ingestion
Head and Neck tumors patients with flushing response/inactive ALDH1 allele
Gastric cancer
Hereditary diffuse gastric cancer
Lynch syndrome
Peutz-Jeghers syndrome
Juvenile polyposis syndrome
Li-Fraumeni syndrome
Atrophic gastritis/pernicious anemia
Post-partial gastrectomy
Sporadic adenoma
18-60 yr old Inhabitants of high-risk areas
Pancreatic cancer
Hereditary pancreatitis
Peutz-Jeghers syndrome
Familial pancreatic cancer kindred (≥ 1 first-degree relative and ≥ 3 first, second or third degree relative with pancreatic cancer)
Familial atypical multiple mole melanoma
Familial breast-ovarian cancer
Hereditary nonpolyposis colorectal cancer (Lynch syndrome)
Familial adenomatous polyposis (FAP)
Cystic fibrosis
Fanconi anemia
Ataxia telangiectasia
Incidentally discovered IPMN/PanIN lesions
Hepatocellular carcinoma
Hepatitis B carriers (Asians and Africans)
Hepatitis B cirrhosis
Family history of HCC (mainly Asians and Africans)
Treated hepatitis B cirrhosis (Asians)
Hepatitis C cirrhosis
Treated hepatitis C cirrhosis
Alcoholic cirrhosis
Genetic hemochromatosis
Alfa1-antitrypsin deficiency
Primary biliary cirrhosis
Colorectal cancer
Familial adenomatous polyposis
Attenuated FAP (AFAP)
Hereditary nonpolyposis colorectal cancer (Lynch syndrome)
Peutz-Jeghers syndrome
Juvenile polyposis syndrome
MUTYH-associated polyposis
Hyperplastic polyposis
Patients with long-standing IBD
Acromegaly patients
Positive findings on index colonoscopy (at 50 yr) such as three or more tubular adenomas, tubular adenoma > 10 mm, adenoma with villous histology, adenoma with high-grade dysplasia, after surgical removal of invasive cancer, incomplete removal of neoplastic lesion

IPMN: Intraductal papillary mucinous neoplasms; PanIN: Pancreatic intraepithelial neoplasia; HCC: Hepatocellular carcinoma; IBD: Inflammatory bowel disease; ALPH1: Aldehyclen dehydrogenase 1; MUTYH: Human MutY homolog.

sociated with any kind of familial predisposition and even lesser proportions (< 5%) belong to well-defined inherited syndromes (such as Lynch syndrome, FAP *etc*.[84]), screening colonoscopy at age 50 (also called index colonoscopy) is the main tool of CRC risk-stratification in general[85]. Presence of adenomas on index colonoscopy is a strong predictor of the risk of development of additional adenomas (30%-50% detection rate at follow up after clearance colonoscopy[86]) and CRC in future. However, most adenomas don't progress to cancer (the life-time cumulative incidence of CRC is 5.5%, and prevalence of colonic adenomas at age 60 is 30%-40%[87]), and therefore their size, numbers, morphology and histopathological characteristics are used to assess the relative risk of progression to cancer and the need of follow-up surveillance/treatment strategies.

In individuals with familial predisposition, the average life-time risk of CRC varies from 100% in FAP to 20% in persons with first and/or second degree relatives with CRC[88], due to difference in penetrance of the inherited genetic defects. It is estimated that only 5% of CRC cases are associated with highly penetrant inherited mutations with well-characterized clinical presentation such as FAP, Lynch syndrome *etc*. whereas rest belong to less penetrant but far more common genetic defects such a polymorphisms in CYP450 family, glutathione-S-transferase family, insulin-like growth factor binding protein-3, ornithine decarboxylase-1 and transforming growth factor-beta receptor 1 genes[84]. However, currently the genetic testing is recommended only if a well-characterized familial syndrome (e.g., Lynch syndrome, FAP) is suspected, because the epidemiological data of the relative-risk of CRC associated with gene polymorphisms is still limited.

Two other well-established high-risk cohorts where surveillance for CRC is recommended are patients with long standing inflammatory bowel disease (IBD) and acromegaly patients. Recent reports suggest that both ulcerative colitis and Crohns' disease patients are at comparable cumulative risk of CRC if the extent and duration of the disease are the same (in case of ulcerative colitis, risk of CRC stands at 1.6% at 10 years, 8.3% at 20 years and 18.4% at 30 years[89,90]). For IBD patients with colonic disease, screening is recommended after 10 years of disease history, and involves endoscopic evaluation of inflammatory changes in the mucosa combined with multiple biopsies to detect dysplasia[91]. Acromegaly patients, on the other hand, seem to have increased incidence as well as propensity for malignant transformation of adenomas, especially right-sided ones, as compared to the general population (odds ratio: 2.4 for adenoma, 7.4 for CRC[92]). This could possibly be attributed to the presence of elevated serum insulin growth factor-1 level (seen in > 90% of acromegaly patients), which has been shown to increase the risk of CRC in non-acromegalic population[93]. Additionally, colonoscopic screening starting at age 40 is recommended to detect precursor lesions in such patients.

From a screening test perspective, non-invasive screening tests such as stool tests (occult blood and DNA tests), imaging (CT, Barium enema) and sigmoidoscopy are only occasionally used, and a full-length colonoscopy despite its several limitations remains the most effective and preferred screening test for CRC (reviewed in details in reference[85]). Current guidelines recommend

screening colonoscopy in average risk individuals at age 50, a significant deviation from earlier practice of colonoscopic screening only in high-risk individuals[94]. The rationale for colonoscopic surveillance has always been based on the high detection rate of colorectal adenomas at follow up (30%-50%) after a complete clearance colonoscopy[86]. However, the main object of colonoscopic surveillance is the prevention of subsequent colorectal cancer rather than the detection and removal of adenomas, most of which will not become malignant. Adenomas with advanced pathology (> 1 cm, with villous elements or severe dysplasia) have a much higher malignant potential, and the main objective of screening is to ensure that such lesions are detected before they become invasive. Therefore, individuals with 1-2 small polyps < 1 cm size and no villous morphology at index colonoscopy are considered low-risk and need no modification in surveillance protocol. However, certain findings on index colonoscopy (as mentioned in the Table 1) indicate high-risk of CRC and necessitate enhanced surveillance.

FUTURE PERSPECTIVE

Currently, surveillance for GI malignancies is challenging because of the general lack of inexpensive screening tests and potent biomarkers that could efficiently identify high-risk cohorts. Recently, there has been a surge in interest in using a panel of biomarkers (gene expression signatures) for screening purposes, but their impact on cancer mortality remains to be tested in large-scale studies[95,96]. Another new class of biomarkers under investigation these days are miRNAs, a type of non-coding RNAs that are endogenous silencers of target genes[97,98]. Unfortunately, many biomarkers/screening tests with initial promise indeed fail to meet the Early Detection Research Network-outlined criteria for their validation[99], and therefore are not used clinically. From a futuristic perspective, we are standing at the crossroads of a major change in our approach towards cancer prevention. With completion of the human genome project, rapid advances in deep sequencing technology and better understanding of the genetic landscape of different tumors (including GI cancers), it is being expected that it would be possible to assess the cumulative predisposition to different cancers in every individual in a cost-effective manner, leading to a highly individualized treatment and preventive care (Personalized Medicine) in coming years[100].

SEARCH STRATEGY AND SELECTION CRITERIA

References for this review were identified through searches of PubMed with the following search terms: "Gastrointestinal malignancies", "risk stratification", "gastrointestinal cancer screening/surveillance", "cancer biomarker", and "cancer prevention" published before December 2011. Articles were also identified through searches of the authors' own files. The final reference list was generated on the basis of relevance to the broad scope of this review; and only articles published in English were reviewed, and considered for inclusion in the reference list based upon the further reading opportunity they offered.

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REVIEW

Management of chronic hepatitis B in pregnancy

Guo-Rong Han, Chuan-Lu Xu, Wei Zhao, Yong-Feng Yang

Guo-Rong Han, Chuan-Lu Xu, Department of Gynecology and Obstetrics, The Second Affiliated Hospital of the Southeast University, Nanjing 210003, Jiangsu Province, China
Wei Zhao, Yong-Feng Yang, Department of Infectious Diseases, The Second Affiliated Hospital of the Southeast University, Nanjing 210003, Jiangsu Province, China
Author contributions: Han GR designed the study and wrote the manuscript with input from the others; Xu CL provided writing assistance; Han GR, Xu CL, Zhao W and Yang YF participated in data collection; Han GR and Xu CL performed the data analysis; and Han GR interpreted the data, wrote the draft and critically revised the manuscript.
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Correspondence to: Guo-Rong Han, MD, Director of the Department of Gynecology and Obstetrics, The Second Affiliated Hospital of the Southeast University, No.1 Zhongfu Road, Nanjing 210003, Jiangsu Province, China. hanguorong2011@sina.com
Telephone: +86-25-83626372 Fax: +86-25-83626060
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Abstract

Pregnancy associated with chronic hepatitis B (CHB) is a common and important problem with unique challenges. Pregnant women infected with CHB are different from the general population, and their special problems need to be considered: such as the effect of hepatitis B virus (HBV) infection on the mother and fetus, the effect of pregnancy on replication of the HBV, whether mothers should take HBV antiviral therapy during pregnancy, the effect of these treatments on the mother and fetus, how to carry out immunization of neonates, whether it can induce hepatitis activity after delivery and other serious issues. At present, there are about 350 million individuals with HBV infection worldwide, of which 50% were infected during the perinatal or neonatal period, especially in HBV-endemic countries. Currently, the rate of HBV infection in the child-bearing age group is still at a high level, and the infection rate is as high as 8.16%. Effective prevention of mother-to-child transmission is an important means of reducing the global burden of chronic HBV infection. Even after adopting the combined immunization measures, there are still 5%-10% of babies born with HBV infection in hepatitis B e antigen positive pregnant women. As HBV perinatal transmission is the main cause of chronic HBV infection, we must consider how to prevent this transmission to reduce the burden of HBV infection. In this population of chronic HBV infected women of childbearing age, specific detection, intervention and follow-up measures are particularly worthy of attention and discussion.

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Key words: Chronic hepatitis B; Hepatitis B virus; Mother-to-child transmission; Perinatal transmission; Pregnancy; Vertical transmission; Antiviral therapy

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INTRODUCTION

Chronic hepatitis B (CHB) in pregnancy is an important and pervasive issue with unique challenges. However, for pregnant women with chronic hepatitis B virus (HBV) infection, unlike the general population, many special

problems need to be considered, such as the influence of HBV infection on the mother and fetus, influence of pregnancy on HBV replication, effects of antiviral treatment on maternal and neonatal outcomes, immunization of newborns and the possible flare of hepatitis after delivery. Approximately 350 million people are infected with HBV worldwide and 50% of them have acquired their infection in the perinatal or neonatal period, especially in countries where HBV has a high prevalence[1,2]. In these countries, women of childbearing age have a higher hepatitis B e antigen (HBeAg)-positive rate and a higher probability of mother-to-infant transmission, the younger they are when infected with HBV, the higher the risk of developing CHB[1,3,4]. The rate of HBV infection among women of childbearing age is still at a high level (7.18%) in China[5-7], which leads to an increased risk of HBV vertical transmission and damage due to CHB in the mother and fetus in pregnancy. Therefore, the effective prevention of vertical transmission of HBV is an important approach in reducing the global burden of CHB. Specific hepatitis B immunoglobulin (HBIG) is available for passive protection and is normally used in combination with hepatitis B vaccine to confer immediate cover (passive immunity) and long-lasting protection (active immunity) in newborns, which is administered as an effective prophylactic measure to prevent mother-to-infant transmission of HBV, however, 5%-10% infants of HBeAg-positive mothers are still infected with HBV[8-10].

PREGNANCY AND CHRONIC HBV INFECTION

Overall, no severe effects due to CHB are found in pregnancy. Studies have shown that chronic HBV infection is associated with gestational diabetes mellitus, antepartum hemorrhage, threatened premature labor and lower Apgar score. Mothers with seriously abnormal liver function complications are prone to postpartum hemorrhage, puerperal infection, low body weight infants, fetal distress, premature birth, fetal death and neonatal asphyxia[11-21]. A series of physiological changes occur during pregnancy, including vigorous metabolism and increased nutrient consumption. These changes occur to promote the metabolic needs of the mother as well as the needs of the growing fetus. Abundant sex hormone produced by the mother needs to be metabolized and inactivated in the liver, and metabolism and detoxification in the fetus also depend on the mother's liver, which correlates with aggravation of pre-existing liver diseases and exacerbation of liver damage[22,23]. Alanine aminotransferase (ALT) in late pregnancy and the postpartum period shows an increasing tendency, however, HBV replication in the gestational period is not noticeably different[24-28]. Some women appear to undergo HBeAg seroconversion in the initial months after delivery if immune activation occurs, with a seroconversion rate of 12.5% to 17%, which is correlated with an obvious decrease in adrenal cortex hormone[29,30]. Although HBV infection during pregnancy can often be tolerated, severe hepatitis and hepatic failure induced by perinatal hepatic flare reactions still occur, and can have an unfavorable outcome[29].

PERINATAL MANAGEMENT OF CHB

As relatively safe assays for the diagnosis of HBV infection are available and effective treatment strategies for CHB have been developed, the screening of HBV infection in the perinatal period has become standard care, which can identify newborns that require prophylaxis with hepatitis B vaccine and HBIG as well as pregnant women who require antiviral therapy. In addition, it is beneficial to advise patients with hepatitis B about sexual and family contacts. Screening and vaccination are key factors in the successful prevention and control of HBV infection.

Women with CHB should actively plan their pregnancy and undergo baseline evaluations, such as hepatitis B surface antigen (HBsAg), HBeAg, antibody to HBeAg (anti-HBe), HBV DNA, severity of liver disease and the presence of other viral infections, are suggested before pregnancy. The ability to sustain pregnancy and the risk of vertical transmission of HBV in women with CHB should also be evaluated. All pregnant women should be screened for HBV infection at the first prenatal examination, and HBsAg-positive patients should be transferred to hospitals with experience in the management of CHB for easier monitoring of mothers in pregnancy, delivery and the postpartum period as well as newborns, and appropriate prevention of mother-to-infant transmission of HBV based on an individual's condition should be conducted.

PREVENTION AND TREATMENT OF CHRONIC HBV INFECTION IN PREGNANCY

The treatment goals for CHB in pregnancy are to achieve stabilization of liver function in mothers and prevent HBV infection in newborns. Regular monitoring of liver function and HBV DNA level should be performed in the gestational period to determine whether liver disease is progressing and antiviral therapy is needed in mothers. Sinha *et al*[11] from India made several suggestions aimed at Asian HBV carriers when planning a pregnancy: First, in patients with lower HBV DNA level at baseline (HBV DNA $< 10^6$ copies in HBeAg-positive patients and HBV DNA $< 10^5$ in HBeAg-negative patients) and no obvious fibration, antiviral therapy may be delayed, but monitoring should be performed during pregnancy. In patients with HBV DNA $> 10^7$ copies/mL repeated in the late trimester of pregnancy, or prior delivery history of a HBV-positive infant and HBV DNA $> 10^6$ copies/mL, antiviral therapy should be administered; Second, in patients with higher HBV DNA level at baseline and obvious fibration, but without liver cirrhosis, antiviral

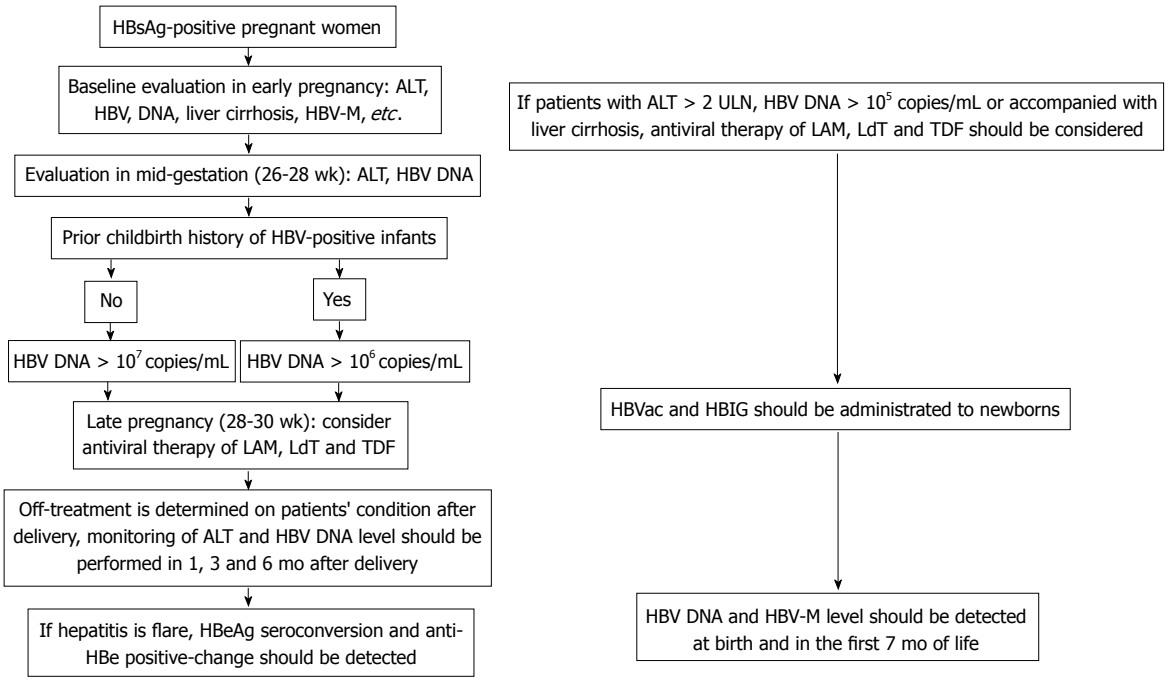


Figure 1 Management of chronic hepatitis B in pregnancy. HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; LAM: Lamivudine; LdT: Telbivudine; TDF: Tenofovir; HBeAg: Hepatitis B e antigen; Anti-HBe: Antibody to HBeAg; ULN: Upper limits of normal; HBVac: Hepatitis B vaccine; HBIG: Hepatitis B immunoglobulin.

therapy is suggested. If a response is sustained after off-treatment, pregnancy is feasible, monitoring should be performed, and management is the same as that outlined in the first scenario; If a response is not sustained after drug withdrawal, management is the same as the third scenario; Third, If patients have liver cirrhosis before pregnancy, antiviral therapy [lamivudine (LAM), tenofovir (TDF) or telbivudine (LdT)] is suggested first, and one of these drugs should be continued during pregnancy, and monitoring should also be conducted.

To effectively prevent HBV infection in infants, delivery methods are also believed to be potential risk factors for mother-to-infant transmission[31,32], however, there is no clear evidence to show that delivery methods are correlated with reduced vertical transmission of HBV[33-35]. Immediate vaccination with HBVac in combination with HBIG in infants born to CHB mothers can effectively prevent infection in the labor and postnatal period, but has no effect on intrauterine infection[36-38], which is the primary cause leading to failure of vaccination. As both HBV intrauterine and perinatal transmission is significantly correlated with HBV DNA level in pregnant women[39-45], currently most attention is focused on oral antiviral drugs in late pregnancy, which reduce HBV intrauterine transmission by decreasing HBV DNA titers in peripheral blood before delivery[46-48].

PROBLEMS CORRELATED WITH ANTIVIRAL THERAPY DURING PREGNANCY

The current difficulty in preventing mother-to-infant transmission of HBV is the prevention of intrauterine transmission. High HBV DNA level is the most important independent risk factor for intrauterine transmission, thus pregnant woman can take oral antiviral drugs in late pregnancy to reduce HBV DNA titers in peripheral blood before delivery and decrease HBV intrauterine transmission.

Selection of antiviral drugs

Due to inhibition of cell proliferation, interferon is contraindicated in pregnant women. For patients using interferon, pregnancy is practicable after discontinuation of interferon for 6 mo. LdT and TDF are authorized category B antiviral drugs by the Food and Drug Administration for use in pregnancy. After reviewing the increasing safety data on LAM in clinical practise[49-52], LAM was elevated to a category B antiviral drug in pregnancy by NIH, that is, the category B antiviral drugs in pregnancy include LAM, LdT and TDF[33,53].

Indication for antiviral therapy

For HBsAg-positive pregnant women by screening, baseline evaluation, such as HBV-M (HBsAg, HBeAg, anti-Hbe), HBV DNA, hepatitis activity and severity of hepatic fibrosis/liver cirrhosis, are suggested in early pregnancy[54-56]. In patients with higher HBV DNA level and hepatitis activity (ALT > 2 upper limit of normal, HBV DNA > 10^5 copies/mL) at baseline or accompanied by liver cirrhosis, antiviral therapy should be administered during early pregnancy. For patients with normal liver function, ALT and HBV DNA level should be reevaluated in mid-gestation (26-28 wk). For patients with HBV DNA > 10^7 copies/mL or prior delivery his-

tory of HBV-positive infants and HBV DNA > 10^6 copies/mL, antiviral therapy (LAM, TDF or LdT) should be given at 28-30 wk until 4 wk after delivery, then it should be determined whether the above therapy is to be continued on the basis of the patient's condition; otherwise antiviral therapy should not be given[57]. ALT and HBV DNA level should be monitored in all HBsAg-positive pregnant women at 1, 3 and 6 mo after delivery. In a hepatitis flare, HBeAg seroconversion and anti-Hbe positive-change should be detected[58]. Active-passive immunization should be performed in all newborns on schedule; HBV-M (HBsAg, HBeAg, anti-HBe) and HBV DNA level should also be detected at birth and in the first 7 mo of life in newborns[59]. In patients with liver cirrhosis before pregnancy, antiviral therapy (LAM, TDF or LdT) are suggested first, one of these drugs should be continued during pregnancy, and monitoring should also be conducted (Figure 1).

Individualized management of CHB in pregnancy

For women with an unplanned pregnancy during the course of antiviral therapy for CHB, individualized management is performed according to the actual condition of the patient. There are two options for patients: one is temporal off-treatment and whole course monitoring of HBV DNA and ALT level, in addition, antiviral therapy is based on the patient's actual condition after pregnancy, which is suitable for patients with mild hepatitis and a lower risk of recurrence and disease progression; the other option is sequential use of LAM, TDF or LdT as antiviral therapy during the whole course[11,36]. Although active-passive immunity should be given to all newborns of HBsAg-positive pregnant women, breast feeding does not increase the risks of HBV infection. However, there is not enough safety data on these drugs with regard to newborn exposure during breast feeding to assess whether patients receiving antiviral therapy should breastfeed their children [57].

Overall, HBV perinatal transmission is a major cause of chronic HBV infection. To reduce the burden of HBV infection, we must consider how to prevent HBV transmission. For women of childbearing age, HBV detection and intervention deserves special attention and investigation.

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ORIGINAL ARTICLE

Loss of fragile histidine triad and amplification of 1p36.22 and 11p15.5 in primary gastric adenocarcinomas

Yuan-Yuan Liu, Hai-Ying Chen, Man-Li Zhang, Dan Tian, Shibo Li, Ji-Yun Lee

Yuan-Yuan Liu, Man-Li Zhang, Dan Tian, Department of Internal Medicine, The First Teaching Hospital of Jilin University, Changchun 130021, Jilin Province, China
Hai-Ying Chen, Department of Infectious Diseases, The First Teaching Hospital of Jilin University, Changchun 130021, Jilin Province, China
Shibo Li, Ji-Yun Lee, Department of Pediatrics, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, United States
Ji-Yun Lee, Department of Pathology, College of Medicine, Korea University, Seoul 136-705, South Korea
Author contributions: Liu YY performed the data analysis; Chen HY performed the data collection; Zhang ML and Tian D collected the samples; Li S designed the study and provided financial support; Lee JY designed the study and wrote the manuscript.
Correspondence to: Ji-Yun Lee, PhD, Department of Pathology, College of Medicine, Korea University, 5-1 Anam-Dong, Seoungbuk-Gu, Seoul 136-705,
South Korea. jiyun-lee@korea.ac.kr
Telephone: +82-2-9206141 Fax: +82-2-9533130
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Abstract

AIM: To investigate the genomic copy number alterations that may harbor key driver genes in gastric tumorigenesis.

METHODS: Using high-resolution array comparative genomic hybridization (CGH), we investigated the genomic alterations of 20 advanced primary gastric adenocarcinomas (seventeen tubular and three mucinous) of Chinese patients from the Jilin province. Ten matching adjacent normal regions from the same patients were also studied.

RESULTS: The most frequent imbalances detected in these cancer samples were gains of 3q26.31-q27.2, 5p, 8q, 11p, 18p, 19q and 20q and losses of 3p, 4p, 18q and 21q. The use of high-resolution array CGH increased the resolution and sensitivity of the observed genomic changes and identified focal genetic imbalances, which included 54 gains and 16 losses that were smaller than 1 Mb in size. The most interesting focal imbalances were the intergenic loss/homozygous deletion of the fragile histidine triad gene and the amplicons 11q13, 18q11.2 and 19q12, as well as the novel amplicons 1p36.22 and 11p15.5.

CONCLUSION: These regions, especially the focal amplicons, may harbor key driver genes that will serve as biomarkers for either the diagnosis or the prognosis of gastric cancer, and therefore, a large-scale investigation is recommended.

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Key words: Array comparative genomic hybridization; Amplicon; Gastric adenocarcinoma; Oncogene; Fragile histidine triad

Peer reviewers: Hitoshi Tsuda, MD, PhD, Diagnostic Pathology Section, Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; Dr. Debra F Saxe, Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA 30322, United States

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INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related death worldwide. Although its incidence has gradu-

ally decreased in many Western countries, the incidence of gastric cancer still remains high in South and Central America and is highest in Eastern Asia, specifically in China, South Korea and Japan[1,2]. The most common gastric malignancy is adenocarcinoma[3], which is characterized by multiple genetic instabilities, as are other adenocarcinomas. One of these genetic instabilities is chromosomal instability, a common consequence of a chromosomal or chromosome-segment abnormality, that causes DNA copy number changes during tumor progression. These alterations may lead to a loss of function of tumor suppressor genes (inactivation) and/or a gain of function of oncogenes (activation). High-level DNA copy number changes (amplification/amplicons) in tumors are frequently restricted to certain chromosomal regions containing well-known oncogenes that are also overexpressed or activated[4,5]. Some oncogenes, such as *NMYC*, *LMYC* and *GLI*, were originally discovered because of their genomic amplification in human tumors[4]. An analysis of the composition of DNA amplifications showed that human cancer can be classified *via* DNA copy number profiling because such amplifications are non-randomly selected with respect to the biological backgrounds of cancer[6]. Therefore, the detection and discovery of unidentified or incompletely described amplicons and relevant genes located within these amplicons can lead to the identification of genes putatively involved in growth control and tumorigenesis.

Recently available whole-genome array comparative genomic hybridization (CGH), a high-throughput genomic technology, facilitates the accumulation of high-resolution data of genomic imbalances associated with disease. In this study, we identified possible candidate genes that could provide insight into the pathology of gastric adenocarcinoma through the integration of genomic copy number changes.

MATERIALS AND METHODS

Tumor samples

This study included seventeen tubular and three mucinous adenocarcinomas of advanced primary stomach cancer samples from Jilin Province in North-Eastern China (Table 1). Of the twenty samples studied, thirteen were from males and seven were from females. The mean age was 62.1 (ranging from 52 to 76) years. The stage of each tumor was classified according to the tumor node metastasis classification of the International Union Against Cancer. The histopathological grades were as follows: Grade 1 (well-differentiated/low grade adenocarcinoma), no cases; Grade 2 (moderately differentiated/intermediate-grade adenocarcinoma), five (tubular) cases; and Grade 3 (poorly differentiated/high-grade adenocarcinoma), 15 (12 tubular and three mucinous) cases. Unfortunately, we were unable to obtain information concerning postsurgical pathological stages. Tumor samples were obtained surgically in the First Hospital of Jilin University; paired adjacent normal tissue was also collected as a control for comparison with the tumor. All patients had negative histories of exposure to either chemotherapy or radiotherapy before surgery, and there were no other diagnosed cancers. An informed consent with approval of the ethics committee of the First Hospital of Jilin University was obtained from all participating patients.

Twenty tumor samples and ten paired adjacent normal tissues were snap-frozen after surgical resection and stored at -80 ℃. DNA was isolated from the tumor tissue by proteinase K digestion followed by phenol-chloroform extraction according to standard protocols.

Array CGH assay

Array CGH was performed according to the manufacturer's protocol with minor modifications on a 385k oligonucleotide chip (Roche/NimbleGen Systems Inc., Madison, WI). Commercially available pooled normal control DNA (Promega Corporation, Madison, WI) was used for reference. The patient DNA and the reference DNA were labeled with either cyanine 3 (Cy-3) or cyanine 5 (Cy-5) by random priming (Trilink Biotechnologies, San Diego, CA) and then hybridized to the chip *via* incubation in the MAUI hybridization system (BioMicro Systems, Salt Lake City, UT). After 18-h hybridization at 42 ℃, the slides were washed and scanned using a GenePix 4000B (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Profile smoothing and breakpoint detection were performed with NimbleScan version 2.4 and SignalMap version 1.9 (NimbleGen Systems). If a smoothed copy number $\log_2$ ratio was above 0.15 or below -0.15 across five neighboring probes, it was defined as a gain or a loss, respectively. Amplifications were defined as those with a smoothed DNA copy number ratio above 0.5.

RESULTS

Overview of genomic imbalances in 20 primary gastric adenocarcinomas

An overview of genomic imbalances in the twenty advanced primary gastric adenocarcinomas is shown in Figure 1. Genomic copy number changes (gains, losses, amplifications or homozygous deletions) were detected in all cases except one. Net gains (15 cases) of genetic material were more frequent than net losses (4 cases). The sizes of the net genomic imbalances per sample ranged from a loss of 122.2 Mb (4.1% of genome) to a gain of 336.9 Mb (11.2% of genome) (Table 1). The mean number of gains per case was 9.0, ranging from 0 to 40, and the mean number of losses per case was 3.5, ranging from 0 to 14. The gain sizes ranged from 56.3 kb to 158.6 Mb, and the loss sizes ranged from 150.1 kb to 131 Mb. Approximately 28% (70/250) of the genomic imbalances were smaller than 1 Mb; from this subset, 21.6% (54/250) of the total imbalances were gains, and 6.4% (16/250) were losses. The most frequent

Table 1 Clinical characteristics, risk factors and overall genomic imbalances in 20 gastric adenocarcinomas

No.	ID	Sex/age (yr)	T/N/M stage	Tumor type	Histology grade (differentiated)	Tumor location	Smoke history	Drink history	Genomic size of total gain (Mb)	Genomic size of total loss (Mb)	Net imbalances (Mb) (%)[2]
1	T64	M/75	T3/N3/M0	Tubular	Poorly	Upper (CR), lower (AA)	Y	Y	290.7	147.3	+143.4 (4.8)
2	TW0800	M/65	T3/N3/M0	Tubular	Poorly	Upper (CR/GF), central (GB)	N	N	388.9	205.3	+183.6 (5.6)
3	T74	M/58	T3/N3/M0	Tubular	Poorly	Central (GB)	N	Y	290.1	412.3	-122.2 (4.1)
4	TW0784	M/75	T3/N1/M0	Tubular	Poorly	Upper (CR/GF)	N	N	3.4	0	+3.4 (0.1)
5	T66	M/50	T3/N2/M0	Tubular	Poorly	Lower (AA)	N	Y	34.2	0	+34.2 (1.1)
6	T78	F/59	T3/N1/M0	Tubular	Poorly	Lower (AA)	N	N	146.2	0	+107.4 (3.6)
7	T41	M/73	T3/N2/M0	Tubular	Poorly	Lower (AA/P)	Y	Y	7.2	0	+7.2 (0.2)
8	T47	F/52	T3/N1/M0	Tubular	Poorly	Lower (AA)	N	N	0.0	0.0	0.0 (0.0)
9	T38	F/53	T2/N1/M0	Tubular	Poorly	Lower (AA/P)	N	N	294.3	37.4	+256.9 (8.6)
10	TW0796	M/56	T3/N1/M0	Tubular	Poorly	Lower (AA/P)	Y	Y	224.4	11.9	+212.5 (7.1)
11	TW0807	M/54	T3/N0/M0	Tubular	Poorly	Lower (AA/P)	Y	Y	191.7	43.7	+148.0 (4.9)
12	TW0813	F/57	T3/N2/M0	Tubular[1]	Poorly	Lower (AA/P)	N	N	76.1	54.6	+21.5 (0.7)
13	T52	M/73	T3/N2/M0	Tubular	Moderately	Upper (CR/GF)	N	N	333.9	361.8	-27.9 (0.9)
14	TW0797	M/62	T3/N1/M0	Tubular	Moderately	Upper (CR/GF)	N	Y	161.1	0	+161.1 (5.4)
15	TW0782	M/53	T3/N2/M0	Tubular	Moderately	Upper (CR/GF)	Y	N	290.1	108.3	+181.8 (6.1)
16	TW0780	F/59	T2/N0/M0	Tubular	Moderately	Lower (AA/P)	N	N	4.1	4.16	-0.06 (0.0)
17	T75	M/69	T3/N2/M0	Tubular	Moderately	Lower (AA)	Y	Y	28	0	+28.0 (0.9)
18	T76	F/59	T3/N1/M0	Mucinous	Poorly	Upper (CR/GF)	Y	Y	99.1	0	+99.1 (3.3)
19	TW0789	M/64	T3/N1/M0	Mucinous	Poorly	Lower (AA/P)	N	N	504.5	167.6	+336.9 (11.2)
20	TW0774	F/76	T3/N3/M0	Mucinous	Poorly	Lower (AA/P)	N	N	41.4	90.4	-49.0 (1.6)

[1]Signet ring cell; [2]Percent of net imbalances calculated based on 3000 Mb of genome size. F: Female; M: Male; T: Tumor; N: Node; M: Metastasis ; CR: Cardiac region; GF: Gastric fundus; GB: Gastric body; AA: Antral area; P: Pylorus; Y: Yes; N: No.

genomic imbalances detected in these cancer samples were gains of 3q26.31-q27.2 (6/20), 5p (5/20), 8q [12/20: 8q22.2-q22.3 (10), 8q24.13-q24.22 (12)], 11p (6/20), 18p (4/20), 19q (8/20) and 20q (8/20) and losses of 3p14.2 (6/20), 4p15.1 (6/20), 18q21.2-q22.1 (4/20) and 21q21.1-q21.2 (4/20). However, no genomic imbalances were detected in the ten paired adjacent tissues, demonstrating that these genomic imbalances are tumor related.

Genomic regions with amplification: Possible diagnostic marker loci

The most prominent feature in this study was the amplicons of 11q13 (two tubular and one mucinous), 18q11.2 (three tubular) and 19q12 (three tubular), as well as the novel amplicons 1p36.22 (two tubular) and 11p15.5 (three tubular) (Table 2 and Figure 2). Amplification of 11q13 had the smallest region of overlap (SRO) of 343.7 kb, which included the *CCND1*, *ORAOV1* and *FGF19* genes. Amplification of 18q11.2 had an SRO of 625.0 kb, which included the *GATA6* and *CTAGE* genes. Amplification of 19q12 had an SRO of 1.4 Mb, which included nine genes: *LOC148145*, *LOC10050583*, *UQCRFS1*, *LOC284395*, *VSTM2B*, *POP4*, *PLEKHF1*, *C19orf12* and cyclin E1 (*CCNE1*). The SRO of the novel amplification 1p36.22 was 418.7 kb and included *FEX14*, *CASZ1*, *C1orf127* and *TARDBP*. The SRO of the other novel amplification, 11p15.5, was 343.7 kb and included *MUC5B*, *LOC255512*, *TOLLIP*, *BRSK2*, *HCCA2*, *DUSP8* and *LOC338865*. Other regions in which amplification was detected were 8p23.1, 8q24.21, 10q26.12q26.13, 11q13, 12p12.1, 12q15, 17q12 and 20q13.2; these regions are summarized in Table 2.

The most common losses involved the fragile histidine triad/FRA3B and PDCH7 genes

Six cases had a loss of the fragile histidine triad (*FHIT*) gene, which maps to the common fragile site 3p14.2. Five of these were intergenic losses of the *FHIT* gene,

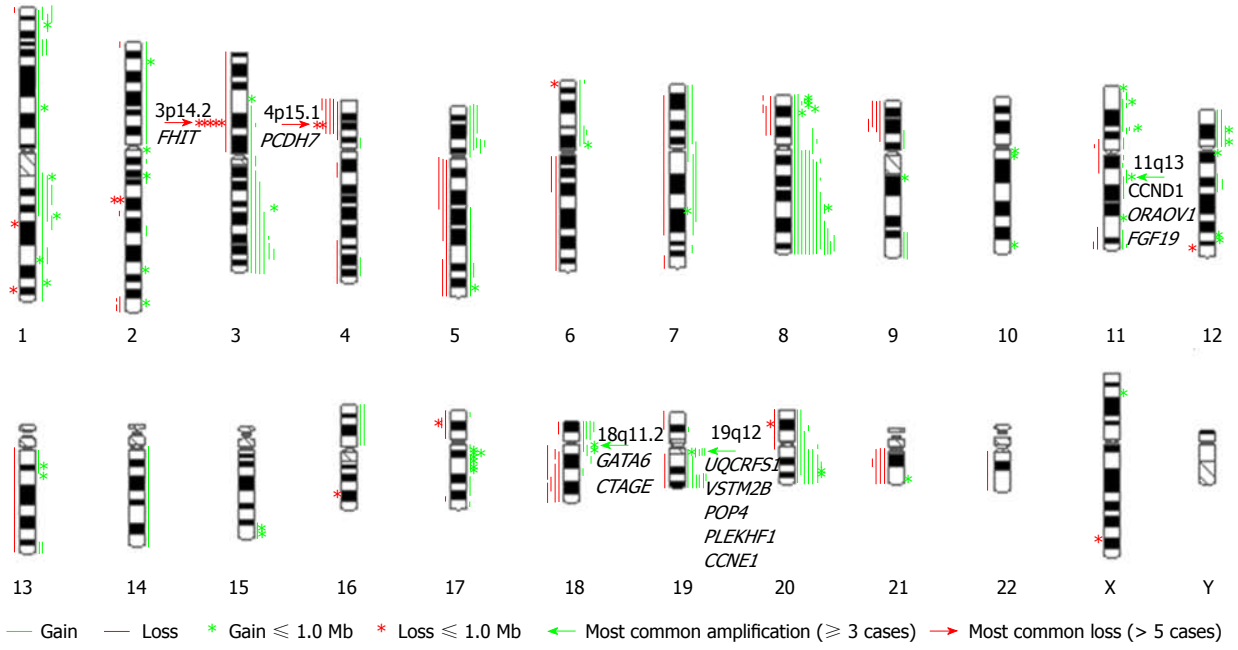


Figure 1 An overview of genomic imbalances in 20 primary gastric adenocarcinomas. The total number of gains was 180 (54 ⩽ 1 Mb), and the total number of losses was 70 (16 ⩽ 1 Mb). Partial or whole gains of 8q (12/20), 19q (8/20), and 20q (8/20) were most frequent. The most common amplicons were of the 11q13, 18q11.2 and 19q12 regions (green arrows). The loss of the *FHIT* gene and the partial loss of 4p with the smallest region of overlap including the *PCDH7* gene were the most frequent losses (red arrow).

and one of these five cases (T38) showed a homozygous deletion. The sizes of these losses ranged from 88.3 kb to 762.5 kb (Table 3 and Figure 3). Another common loss identified in all six cases was the 4p15.1 region (Table 3 and Figure 3). Two cases (TW789 and TW782) had an intergenic loss of the *PDCH7* gene.

DISCUSSION

In this study, we investigated gene/segmental genomic copy number alterations in twenty advanced primary gastric adenocarcinomas (seventeen tubular and three mucinous) *via* whole-genome array CGH. We observed that the total number of gains/amplifications (180) was 2.6 times the total number of losses (70). Nineteen out of the 20 cases had genomic imbalances; fifteen of these had net genomic gains (3.4-336.9 Mb), and four of these had net genomic losses (0.06-122.2 Mb), indicating that genomic gains are more common than losses (Figure 1). These findings are compatible with previous findings determined *via* conventional CGH[7-51]. However, we discovered cryptic genomic imbalances smaller than 1.0 Mb in 28% (70/250) of the total imbalances (with an average of 3.5 per case) and narrowed down the SROs of losses or of gains/amplifications to those including interesting genes or focal genomic segments. These findings are explained by the resolution of the array that we used. The most interesting cryptic imbalances were losses of 3p14.2 and 4p15.1 (Figure 3) and amplifications of 1p36.22, 11p15.5, 11q13, 18q11.2 and 19q12 (Figure 2).

The loss of 3p14.2 in six of our cases encompassed the *FHIT* gene, which was discovered at the FRA3B locus on the short arm of chromosome 3 and is the most active common chromosome fragile site in the human genome. *FHIT*'s loss of function as a tumor suppressor gene due to breakage, allelic loss, occasional homozygous deletion and promoter hypermethylation has been evaluated in different types of epithelial tumors, including gastric cancer, which is strongly associated with direct or indirect exposure to environmental carcinogens[52-54]. The FHIT protein is absent in more than 50% of the observed cases, both in gastric cancer cell lines and in primary gastric adenocarcinomas, irrespective of any specific histotype, indicating that alterations of the *FHIT* gene can play a role in tumorigenicity as an important and preliminary genetic alteration in the cell[55]. However, subsequent additional genetic changes involving other tumor suppressor genes or oncogenes may be necessary for tumor progression. In our six cases who had lost the *FHIT* gene, five showed an intergenic loss of *FHIT*, ranging in size from 150 kb to 762.4 kb. One case had a homozygous deletion of part of the *FHIT* gene (case T36); however, no deletion of this region was found in the normal adjacent tissue of the same case (date not shown). This finding suggests that *FHIT* loss, not normal copy number variation, is clearly linked to tumorigenicity. However, copy number variations, particularly losses including the *FHIT* gene, have been reported in the normal population[56], raising the question of whether constitutional copy number loss of the *FHIT* gene increases tumorigenicity susceptibility.

The other most common loss was of 4p15.1, detected in six cases and encompassing the *PDCH7* gene. PDCH7 is an integral membrane protein that is thought

Table 2 Amplification segments and the genes involved

Chr. region	Amp	Gain	SRO, bp (hg 18)	Size (kb)	Genes	Selected references
1p36.22[1]	2	1	10 587 540-11 006 299	418.7	*PEX14, CASZ1, C1orf127, TARDBP*	N/A
1q21.2	1	3	148 737 723-149 062 575	324.9	*TARS2, ECM1, ADAMTSL4*[3], *MCL1*[3], *ENSA*[2], *GOLPH3L, HORMAD1*[3], *CTSS, CTSK, ARNT*[2]	Gastric cancer[7], adenocarcinoma of the gastroesophageal junction[8][3], basal/luminal breast cancer[9][3], hepatocellular carcinoma[10]
8p23.1	2	3	10 475 239-10 562 632	87.4	*RP1 L1*	Gastric cancer[7,11], esophageal adenocarcinoma[12,13][3],[14],[15][3], adenocarcinomas of the gastroesophageal junction[16][3], small bowel adenocarcinoma[17]
			10 681 251-10 943 920	262.7	*PINX1, XKR6*	
			11 250 228-11 887 602	637.4	*TDH, FAM167A, BLK, GATA4*[2,3,4], *NEIL2*[2], *FDFT1*[2,3], *CTSB*[2,3]	
8q24.21	1	11	128 331 422-128 837 626	506.2	*POU5F1B, LOC727677, MYC*[2,3]	Various cancer[5], esophageal adenocarcinoma[14], gastric cancer[18],[19][2],[20], papillary renal cell carcinoma[21][3]
10q26.12	1	0	121 881 486-123 931 430	2050.0	*PPAPDC1A, LOC283089, WDR11, FGFR2*[3], *ATE1*[3], *NSMCE4A*[3], *TACC2*[3]	Breast cancer[22][3], gastric carcinoma[20]
10q26.13			126 212 750-126 362 686	149.9	*LHPP, FAM53B*	
11p15.5[1]	1	0	1 175 114-1 556 281	381.2	*MUC5B, LOC255512, TOLLIP, BRSK2, HCCA2, DUSP8, LOC33865*	N/A
11p13	2	1	34 675 094-35 068 916	393.8	*APIP*[3], *PDHX*[3]	Breast cancer[23], gastric cancer cell line[24], head and neck squamous cell carcinoma cell line[25][3]
11q13.2q13.3	3	0	68 912 663-69 256 388	343.7	*CCND1*[2,3], *ORAOV1*[2,3], *FGF19*[3]	Various cancers[5], gastric cancer[7,11,20],[26][2], hepatocellular carcinoma[27][2], esophageal adenocarcinoma[14], esophageal and gastric cancer[26], esophageal squamous cell carcinoma[28][3], laryngeal/pharyngeal cancer[29][3]
12p12.1	2	2	25 150 060-25 437 736	287.7	*LRMP*[3], *CASC1, LYRM5, KRAS*[2,3]	Various cancers[5], esophageal adenocarcinoma[14], gastric cancer[11,20],[30][2], ovarian cancer[31][3]
12q15	2	0	67 475 003-67 875 102	400.1	*MDM2*[2], *CPM*	Various cancers[5], esophageal adenocarcinoma[14], gastric cancer[20,32], liposarcomas[33][3], melanoma cell line[34][3]
			68 037 717-68 318 830	281.1	*YEATS4 (GAS41)*[3], *FRS2*[3], *CCT2*[3], *LRRC10*	
17q12	2	1	35 000 176-35 150 077	149.9	*NEUROD2, PPP1R1B*[2], *STARD3*[2], *TCAP, PNMT*[2], *PERLD1*[2], *ERBB2*[2], *C17orf37, GRB7*[2]	Various cancers[5], gastric cancer[11,20],[35-37][2],[38],[39][2]
18q11.2	3	2	17 800 202-18 425 167	625.0	*GATA6*[3,4], *CTAGE1*	Pancreatic carcinoma[40,41][3], esophageal adenocarcinoma[14],[42][3]
19q12	3	3	33 606 476-35 037 396	1340.9	*LOC148145, LOC10050583, UQCRFS1*[2], *LOC284395, VSTM2B, POP4*[2], *PLEKHF1*[2], *C19orf12*[2], *CCNE1*[2]	Gastric cancer[43][2],[44],[45][2], esophageal/gastric cardiac adenocarcinoma[12]
20q13.2	1	6	51 568 862-51 993 797	424.9	*ZNF217*[3], *SUMO1P1, BCAS1*[3]	Esophageal adenocarcinoma[14], adenocarcinoma of the gastroesophageal junction[46][3], breast cancer[47], gastric adenocarcinoma[48], glioblastoma[49], various cancers[50]

[1]Novel amplicon; [2]Gene and references that are overexpressed when amplified in gastric cancer; [3]Genes (and references) that are overexpressed when amplified in types of cancer other than gastric cancer; [4]Underexpression has been reported in gastric cancer. SRO: Smallest region of overlap; N/A: Not available.

to function in cell-cell recognition and adhesion. Loss of heterozygosity (LOH) or deletion of this region has been reported in hepatocellular carcinoma (HCC) and in some head and neck squamous cell carcinomas[57,58]. Recently, a genome-wide analysis revealed a single-nucleotide polymorphism in *PDCH7* whose risk allele affects overall survival in early-stage non-small-cell lung cancer[59].

Gene/chromosomal segment amplifications are thought to reflect genetic instabilities in solid-tumor cells[60]. Such amplifications commonly consist of double minutes (DMs) or homogeneous staining regions or are dispersed at the genomic level; they are usually correlated with protein levels of genes[61]. It has been proposed that the activation of proto-oncogenes by amplification plays an important role in the development of many human solid tumors. Therefore, detection of specific gene amplifications in tumor cells can lead to the identification of genes putatively involved in growth control and tumorigenesis. In our study, we identified the novel amplicons 1p36.22 and 11p15.5 as well as prominent amplicons 11q13, 18q11.2 and 19q12 (≥ 3 cases with amplification).

LOH or the loss of the short arm of chromosome 1, which includes band p36, has been reported in vari-

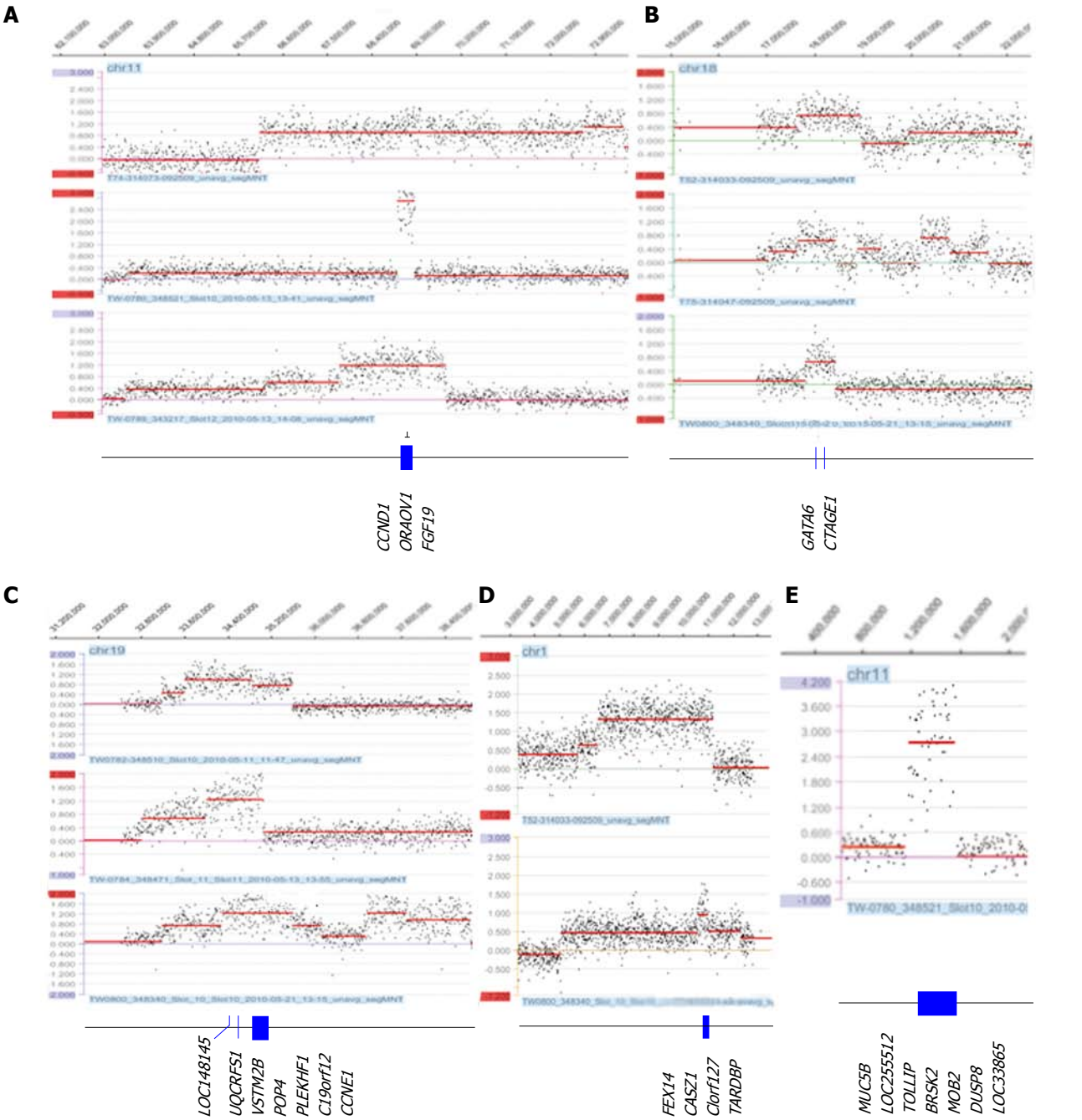


Figure 2 Representative amplifications detected by array comparative genomic hybridization and the genes that are located in the smallest region of overlap. The most common amplicons at 11q13 (A), 18q11.2 (B) and 19q12 (C) and novel amplicons at 1p36.22 (D) and 11p15.5 (E) region (log2 > 0.5). The x-axis indicates the genomic location, and the y-axis indicates the log2 ratio.

ous cancers[62], supporting the possibility that this region encompasses at least one tumor suppressor gene, as opposed to one or more oncogenes. The *CASZ1* gene on 1p36, which was amplified in our cases, has been implicated as a candidate tumor suppressor gene in neuroblastomas[63]. However, our study revealed three cases with a gain of 1p36.22. Of these, two cases with high-level amplification of a 418.7 kb SRO, which includes the *PEX14*, *CASZ1*, *C1orf127* and *TARDBP* genes, had not previously been reported. Further studies could determine whether this amplification induces overexpression of proteins. The other novel amplicon, found in one case, was the 11p15.5 region, which is 343.7 kb and includes the *MUC5B*, *TOLLIP*, *BRSK2*, *HCCA2*, *DUSP8* and *LOC33865* genes. The *MUC5B* gene encodes a member of the mucin family of proteins, which are highly glycosylated macromolecular components of mucus secretions. This family member is the major gel-forming mucin in mucus. The expression of this gene has been associated with a type of gastric carcinoma but not with the clinical-biological behavior of the tumors[64,65]. The *TOLLIP* gene encodes a ubiquitin-binding protein that interacts with several toll-like receptor (TLR) signaling cascade components. The TOLLIP

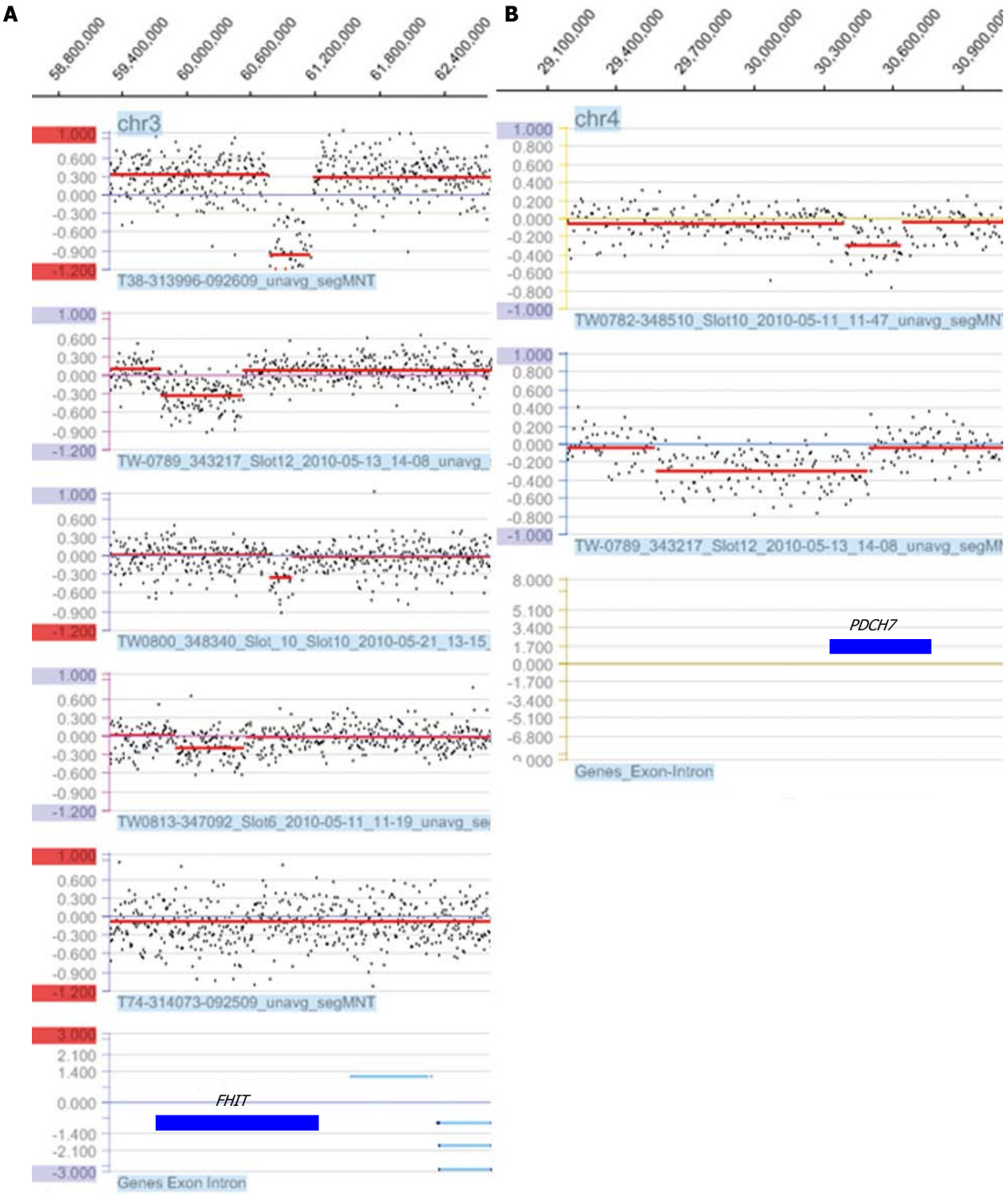


Figure 3 Intergenic loss of *FHIT*/FRA3B on 3p14.2 (A) and *PDCH7* on 4p15.1 (B) detected by array comparative genomic hybridization. The x-axis indicates the genomic location, and the y-axis indicates the log2 ratio.

protein regulates inflammatory signaling and is involved in interleukin-1 receptor trafficking and the turnover of the IL1R-associated kinase; a possible association with human cancer development has been suggested[66]. The BR serine/threonine kinase 2 (*BRSK2*) gene acts as a checkpoint kinase upon DNA damage induced by UV irradiation or methyl methane sulfonate[67]. Clinical implications of *BRSK2* expression in pancreatic ductal adenocarcinoma have been suggested[68]. The hepatocellular carcinoma-associated gene 2 (*HCCA2*) gene was initially identified as a HCC-specific protein. It was subsequently found to interact with *MAD2L2* and might function in cell cycle regulation[69]. The dual specificity phosphatase 8 (*DUSP8*) gene is a member of the *DUSP* subfamily.

Table 3 Summary of losses of *FHIT* at 3p14.2 and of *PDCH7* at 4p15.1

Gene	Chromosome region	Case	Genomic coordinates (NCBI build 36.3; hg18)	Size (kb)
FHIT (HD)	3p14.2	T38	60 775 135-61 162 550	387.4
FHIT	3p14.2	TW0789	59 762 657-60 525 130	762.5
FHIT intron	3p14.2	TW800	60 781 394-60 987 631	206.2
FHIT	3p14.2	TW813	59 900 085-60 543 794	437.5
FHIT etc.	3pter-p11.2	T74	37 570-88 331 466	88 300.0
FHIT intron	3p14.1	T782	60 343 805-60 493 871	150.1
PDCH7 etc.	4p16.3-p13	T64	2 431 351-44 331 469	41 900.0
PDCH7	4p15.1	TW789	29 575 024-30 487 701	912.7
PDCH7 etc.	4pter-p14	TW807	191-37 525 019	37 500.0
PDCH7 etc.	4pter-p14	TW774	191-38 356 422	38 400.0
PDCH7	4p15.1	TW782	30 393 830-30 637 746	243.9
PDCH7 etc.	4pter-p14	TW813	191-38 993 759	39 000.0

HD: Homozygous deletion.

DUSPs inactivate their target kinases by dephosphorylating both the phosphoserine/threonine and phosphotyrosine residues. These genes negatively regulate members of the mitogen-activated protein kinase (MAPK) superfamily (MAPK/ERK, SAPK/JNK, p38), which are involved in cellular proliferation and differentiation. The roles of the DUSPs in the regulation of MAPK activities have been highlighted as part of the oncogenic process[70]. Overall, any of the genes in this region are likely to play an important role in the progression of cancer.

The amplification of 11q13 ranged from 343.7 kb to 20.5 Mb and had an SRO of 343.7 kb, which included the *CCND1*, *ORAOV1* and *FGF19* (*BLC6*) genes. The variously sized 11q13.3 amplicon containing *CCND1* is one of the most frequent amplification events in human tumors. Computational genome-wide approaches to identify driver genes have reported *CCND1* as one of the most common somatic focal amplifications in human cancers[5]. As shown in our 11q13 amplification, the *ORAOV1* and *FGH19* genes lie within 15 kb of *CCND1*, and they are invariably co-amplified with *CCND1*. However, the limited data show that their expression levels depend on the type of tumor, indicating that the driver gene can be tissue-type dependent/specific. The co-expression of *FGH19* with or without *CCND1* has been found in HCC, but an absence of *FGH19* expression has been found in breast cancer and oral cancer[27]. Future work can determine whether *FGH19* is co-expressed with *CCND1* in primary gastric adenocarcinoma, even given that recently published whole-genome expression data did not show a significant correlation or co-expression of amplification/expression of *CCND1* and *FGH19*[35,43]. *ORAOV1* overexpression has been found in all amplified HCCs; however, *ORAOV1* does not promote tumorigenicity in $p53^{-/-}$; Myc hepatoblasts, nor is it cooperative with *FGFR1* or *CCND1*[27]. A total of four cases in this study had a gain of the 18q11.2 region. Of these, three cases had amplifications ranging from 625.0 kb to 1.3 Mb with an SRO of 625.0 kb, which includes the *GATA6* and *CTAGE* genes. This 18q11.2 amplification, along with expression and epigenetic studies, supports the oncogenic function of *GATA6* in esophageal carcinoma, colon cancer and pancreatic cancer[40,42,71,72] but not in gastric adenocarcinoma. Upregulation of *GATA6* has been reported in the transition from normal esophageal epithelium to Barrett adenocarcinoma and adenocarcinoma[42,71]. A 0.36 kb amplification including *GATA6* and *CTAGE1* has been found in pancreatic carcinoma[40,41], and dysregulation (overexpression) of *GATA6* contributes to colorectal tumorigenesis and tumor invasion[72]. Moreover, in that study *GATA6* was overexpressed not only in the cases with an amplification of *GATA6* but also in the cases without amplification[72]. However, *CTAGE* was rarely overexpressed, indicating that *GATA6* is the driver in this amplicon[41] and suggesting that *GATA6* overexpression may play a role in the early stages of tumor development. A contradictory result showing underexpression of the *GATA6* gene has been reported in gastric adenocarcinoma[36], demonstrating that the oncogenic function of *GATA6* in gastric adenocarcinoma needs to be investigated. Our observed amplification of 19q12 with an SRO of 1.4 Mb includes the nine genes *LOC148145*, *LOC10050583*, *UQCRFS1*, *LOC284395*, *VSTM2B*, *POP4*, *PLEKHF1*, *C19orf12* and *CCNE1*. This 19q12 amplification has been found in gastric cancer and esophageal adenocarcinoma[12,44,45]. Of these genes, *CCNE1*, an E type cyclin, has traditionally been considered the target of the 19q amplification, which is also one of the most common amplification products in various tumor types[5,12]. However, a comprehensive analysis of the 19q12 amplification in gastric cancer has revealed clustered overexpression of *CCNE1* as well as *UQCRFS1*, *POP4*, *C19orf12* and *RMP*, indicating potential functions of other genes in this region in tumor development[45]. In ovarian cancer, it has been suggested that the *CCNE1* gene is the key driver in this 19q12 amplicon and is correlated with the gain in the 20q11 region that includes the *TPX2* gene[73]. It is not clear whether the *CCNE1* gene is a key driver in other types of tumors, including gastric cancer tumors, because no detailed study has been conducted; it is also possible that this gene is tissue specific, as shown in 19q12. However, the gain of the 20q11 region is one of the most

common findings in gastric cancer, and the correlation between 19q12 and 20q11 in gastric cancer needs to be investigated.

Other amplifications found were 1q21.2 (1 amp/3 gains), 8p23.1 (2 amps/3 gains), 8p23.1 (2 amps/3 gains), 8q24.21 (1 amp/11 gains), 10q26.12q26.13 (1 amp), 11p13 (2 amps/1 gain), 12p12.1 (2 amps/2 gains), 12q15 (2 amps), 17q12 (2 amps/1 gain) and 20q13.2 (1 amp/6 gains). We summarized these amplifications and the expression of the gene(s) corresponding to these amplicons in various primary epithelial tumor and cell lines (Table 2). *MCL1* on 1q21.1, *MYC* on 8q24.21, *KRAS* on 12p12.1, *MDM2* on 12q15, *ERBB2* on 17q12 and *ZNF217* on 20q13.2 are well-acknowledged oncogenes in several tumors[5]. Recently published data suggest that the same amplifications do not necessarily induce the same gene expression in different tissue types[27], implying that driver genes can be tissue-type specific and making it necessary to acquire and investigate both amplification and overexpression information for different tumor types, even in the case of well-validated oncogenes.

In summary, the array CGH technique allows for comprehensive, rapid and reliable analysis of the whole genome of primary gastric adenocarcinomas and enables the refined and detailed study of amplifications and regions of recurrent copy number change. This approach makes it possible to identify putative oncogenes and tumor suppressor genes that may deserve further investigation. In this context, we identified candidate target genes/genomic segments of amplification that may help to direct therapeutics against gastric cancer.

COMMENTS

Background

Gastric cancer is one of the leading causes of cancer-related death worldwide. Although its incidence has gradually decreased in many Western countries, the incidence of gastric cancer still remains high in South and Central America and is highest in Eastern Asia, specifically in China, South Korea and Japan. Searching for biomarkers for gastric cancer has proven to be quite challenging.

Research frontiers

Cancer is a genetic disease that involves multiple genetic alterations. Understanding the molecular profile of tumor tissue is crucial for efficiently targeting cancer cells. In this study, the authors identified possible candidate target genes that could provide insight into the pathogenesis of gastric adenocarcinoma through the integration of genomic copy number changes.

Innovations and breakthroughs

Recent reports have highlighted the oncogenic addiction associated with oncogene amplification as a driver gene in carcinogenesis and target treatments. In this research paper, the authors identified novel amplicons that have not been published previously and that could be new target sites for potential diagnosis, prognosis and treatment.

Applications

To learn how these new amplicons are induced and change gene expression, this study may represent a future strategy for therapeutic intervention in the treatment of patients with gastric adenocarcinoma.

Peer review

The paper was designed to be a proof of principle for using cytogenomic microarrays on a larger study to ascertain biomarkers for various gastric neoplasms. The authors did a very thorough analysis of the genetic alterations and of the discussion of the possible genes involved. As claimed, they showed the power of the cytogenomic microarray in detecting common genetic alterations in various gastric tumors. The study was organized in a very clear manner and each common abnormality was compared in a thoughtful manner.

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BRIEF ARTICLE

FOLFIRI regimen in metastatic pancreatic adenocarcinoma resistant to gemcitabine and platinum-salts

Cindy Neuzillet, Olivia Hentic, Benoît Rousseau, Vinciane Rebours, Léïla Bengrine-Lefèvre, Franck Bonnetain, Philippe Lévy, Eric Raymond, Philippe Ruszniewski, Christophe Louvet, Pascal Hammel

Cindy Neuzillet, Olivia Hentic, Vinciane Rebours, Philippe Lévy, Philippe Ruszniewski, Pascal Hammel, Service de Gastroentérologie-Pancréatologie, Hôpital Beaujon, 100 Boulevard du Général Leclerc, 92110 Clichy La Garenne, AP-HP, France
Cindy Neuzillet, Benoît Rousseau, Léïla Bengrine-Lefèvre, Service d'Oncologie Médicale, Hôpital Saint Antoine, 184 Rue du Faubourg Saint-Antoine, 75012 Paris, AP-HP, France
Franck Bonnetain, Unité de Biostatistique et d'Epidémiologie EA 4184, Centre Georges François Leclerc, 1 Rue du Pr Marion, 21079 Dijon, France
Eric Raymond, Service d'Oncologie Médicale, Hôpital Beaujon, 100 Boulevard du Général Leclerc, 92110 Clichy La Garenne, AP-HP, France
Christophe Louvet, Département d'Oncologie Médicale, Institut Mutualiste Montsouris, 42 Boulevard Jourdan, 75014 Paris, France
Author contributions: Neuzillet C, Louvet C and Hammel P designed the study; Neuzillet C, Hentic O, Rousseau B, Bengrine-Lefèvre L, Lévy P, Raymond E, Ruszniewski P, Louvet C and Hammel P performed the research; Rebours V and Bonnetain F analyzed the data; and Neuzillet C and Hammel P wrote the paper.
Correspondence to: Dr. Pascal Hammel, Service de Gastroentérologie-Pancréatologie, Hôpital Beaujon, 100 Boulevard du Général Leclerc, 92110 Clichy La Garenne, AP-HP, France. pascal.hammel@bjn.aphp.fr
Telephone: +33-1-40875653 Fax: +33-1-42703784
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Abstract

AIM: To evaluate the efficacy and safety of the FOLFIRI regimen in patients with metastatic pancreatic adenocarcinoma (PAC) after the failure of gemcitabine and platinum salts.

METHODS: All consecutive patients with histologically confirmed, metastatic PAC and World Health Organization performance status (PS) $\leqslant$ 2 received FOLFIRI-1 [irinotecan 180 mg/m^2 on day 1 and leucovorin 400 mg/m^2 followed by 5-fluorouracil (5-FU) 400 mg/m^2 bolus, then 5-FU 2400 mg/m^2 as a 46-h infusion, biweekly] or FOLFIRI-3 (irinotecan 100 mg/m^2 on day 1 and leucovorin 400 mg/m^2, then 5-FU 2400 mg/m^2 as a 46-h infusion and irinotecan 100 mg/m^2 repeated on day 3, biweekly) after failure of gemcitabine and platinum-based chemotherapies as a systematic policy in two institutions between January 2005 and May 2010. Tumor response, time to progression (TTP), overall survival rate (OS) and grade 3-4 toxicities were retrospectively studied. Subgroup analyses were performed to search for prognostic factors.

RESULTS: Sixty-three patients (52.4% male, median age 59 years) were analyzed. Among them, 42.9% were PS 0, 38.1% were PS 1 and 19.0% were PS 2. Fifty one patients (81.0%) had liver metastases. Before the FOLFIRI regimen, patients had received 1 line (n = 19), 2 lines (n = 39) or 3 lines (n = 5) of chemotherapy. Median TTP obtained with the line before FOLFIRI was 3.9 mo (95% CI: 3.4-5.3 mo). A total of 480 cycles was completed (median: 6 cycles, range: 1-51 cycles). The main reason for discontinuing FOLFIRI was tumor progression (90.3%). Tumor control was achieved in 25 patients (39.7%) (partial response: n = 5, stable disease: n = 20) with FOLFIRI. Median TTP was 3.0 mo (95% CI: 2.1-3.9 mo) and median OS was 6.6 mo (95% CI: 5.3-8.1 mo). Dose adaptation was required in 36 patients (57.1%). Fifteen patients (23.8%) had grade 3-4 toxicities, mainly hematological (n = 11) or digestive (n = 4). Febrile neutropenia occurred in 3 patients. There was no toxic death. PS 2 was significantly associated with poor TTP [hazard ratio (HR): 16.036, P < 0.0001] and OS (HR: 4.003, P = 0.004).

CONCLUSION: The FOLFIRI regimen had an acceptable toxicity and an interesting efficacy in our study, limited to patients in good condition (PS 0-1).

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Key words: Pancreatic cancer; Pancreatic adenocarcinoma; Metastases; Chemotherapy; 5-fluorouracil; Irinotecan; Camptothecin; FOLFIRI regimen

Peer reviewer: Dr. Michel M Murr, MD, Director of Bariatric Surgery, Tampa General Hospital, 1 Tampa General Circle, Tampa, FL 33647, United States

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INTRODUCTION

Pancreatic adenocarcinoma (PAC) accounts for 2%-3% of all cancers but is the fourth leading cause of cancer death in Western countries[1]. More than 80% of patients present with unresectable disease and most of those with operable tumors who undergo resection have local relapse or metastases[2]. The overall prognosis of metastatic PAC remains poor, with a 5-year survival rate of less than 5%[3].

Gemcitabine became the reference regimen as first-line chemotherapy in patients with metastatic PAC after a randomized trial showed significant improvement in median overall survival (OS) compared with 5-fluorouracil (5-FU) (5.6 mo *vs* 4.4 mo, $P = 0.002$)[4]. Over the past decade, multiple phase Ⅱ and Ⅲ studies have attempted to improve these results using various combinations of gemcitabine with other agents but no significant benefit on survival has been found compared with gemcitabine alone, except for erlotinib which resulted in a modest but significant improvement in OS (6.2 mo *vs* 5.9 mo, $P = 0.038$)[5,6]. A phase Ⅲ trial comparing the FOLFIRINOX regimen (folinic acid/5-FU, irinotecan and oxaliplatin combination) to gemcitabine as first-line treatment for metastatic PAC showed that this combination was superior to gemcitabine (OS: 11.1 mo *vs* 6.8 mo, $P < 0.001$)[7].

In clinical practice, about half of metastatic PAC patients with disease progression under gemcitabine treatment remain in acceptable clinical condition and thus may receive subsequent line(s) of chemotherapy. A retrospective series of 117 patients evaluated the feasibility and benefits of second- and third-line chemotherapies in patients with metastatic PAC after the failure of gemcitabine[8]. Fifty three (45%) received two lines and 24 (21%) received three or more lines. Median time to progression (TTP) and OS from the beginning of the second line were 2.3 mo and 4.7 mo, respectively. The FFCD 0301 phase Ⅲ trial was the first randomized study to evaluate a chemotherapy strategy with a second line of treatment in the treatment plan. It compared the combination of folinic acid/5-FU and cisplatin followed by gemcitabine or the reverse sequence in metastatic PAC[9]. The second line of therapy was administered at disease progression to 68% of patients who received folinic acid/5-FU and cisplatin as a first line treatment and to 55% in the gemcitabine arm (non-significant). Median progression-free survival (PFS) and OS in the two arms were not significantly different. Although there is no standard regimen in this setting, two randomized studies have indicated that the combination of folinic acid/5-FU and oxaliplatin appeared to be superior to both best supportive care (4.9 mo *vs* 2.3 mo, $P = 0.008$) and folinic acid/5-FU alone (6.0 mo *vs* 3.0 mo, $P = 0.014$) as a second line of treatment[10]. Other regimens have been tested in non-randomized phase Ⅱ studies, but the samples were small and information on World Health Organization (WHO) performance status (PS) and disease stage were often lacking[11].

Preclinical studies have shown that the camptothecin analog irinotecan has significant activity in both cultured pancreatic tumor cells and in xenograft models[12,13]. Irinotecan monotherapy in previously untreated PAC patients yielded response rates (RR) of 9%-27%[14-16]. *In vitro* studies have suggested that there is a synergistic effect between irinotecan and 5-FU[17-19]. A multicenter phase Ⅱ study with folinic acid/5-FU and irinotecan day 1/day 3 combination (FOLFIRI-3 regimen) showed promising activity in chemotherapy-naive patients with locally advanced or metastatic PAC, with a median PFS and OS of 5.6 mo and 12.1 mo, respectively, and a manageable toxicity profile[20]. A randomized phase Ⅱ study has compared modified FOLFIRI-3 and a modified FOLFOX schema (folinic acid/5-FU and oxaliplatin combination) as second-line chemotherapy in that setting[21]. The efficacy was similar, with 6-mo OS rate of 27% and 30%, respectively. An Italian group reported a retrospective series of 40 patients with gemcitabine-resistant locally advanced or metastatic PAC treated with a standard FOLFIRI (FOLFIRI-1, folinic acid/5-FU and irinotecan day 1 combination) regimen[22]. Median TTP was 3.7 mo and median OS was 6 mo.

Because no data exist on the efficacy of the FOLFIRI regimen after the failure of both gemcitabine and platinum salts, we performed a retrospective study to evaluate the efficacy and safety of this regimen in patients with advanced PAC in that setting. As locally advanced PAC may have a more favorable natural history than metastatic PAC, we decided to exclude locally advanced PAC patients from the study to have a homogeneous population.

MATERIALS AND METHODS

Patients

All patients with histologically confirmed, metastatic PAC, after failure (progression or major toxicity) of gemcitabine and platinum-based chemotherapies, re-

ceived an irinotecan-based regimen as a systematic policy after discussion during a weekly multidisciplinary meeting in our institutions (Saint Antoine Hospital, Paris and Beaujon Hospital, Clichy), if they met the following criteria: previous treatment with gemcitabine and platinum salt (combined or given in consecutive lines); WHO PS ≤ 2; at least one bidimensionally measurable lesion according the Response Evaluation Criteria in Solid Tumors (RECIST); absence of severe uncontrolled cardiovascular, metabolic, infectious or renal disease; serum bilirubin level < 1.5 times the upper limit of normal; polynuclear neutrophil count > 1500/mm^3; platelet count > 100 000/mm^3.

Chemotherapy regimen

The FOLFIRI-1 regimen consisted of irinotecan 180 mg/m^2 administered as a 90-min infusion on day 1, together with leucovorin 400 mg/m^2 for 2 h followed by an 5-FU 400 mg/m^2 bolus, then a 46-h infusion of 5-FU 2400 mg/m^2. The FOLFIRI-3 regimen consisted of irinotecan 100 mg/m^2 administered as a 60-min infusion on day 1, together with leucovorin 400 mg/m^2 for 2 h, then a 46-h infusion (without bolus administration) of 5-FU 2400 mg/m^2 and irinotecan 100 mg/m^2 repeated on day 3 at the end of 5-FU infusion. Only FOLFIRI-3 (intensified) regimen has been evaluated in phase II studies in PAC[20,21]. However, the FOLFIRI-1 regimen is extensively used in clinical practice for treatment of other gastrointestinal cancers and seems to be less toxic. Thus, the choice between the FOLFIRI-1 or FOLFIRI-3 regimen was left up to the discretion of the investigator. The chemotherapy cycles were repeated every two weeks if the clinical and biochemical assessment was compatible (as mentioned above).

Patients who developed a cholinergic syndrome received preventive treatment with atropine (0.25 mg subcutaneously) during all subsequent cycles. Late-onset diarrhea was treated using high-dose loperamide. When severe neutropenia occurred and/or did not recover to grade ≤ 1 on day 14, a granulocyte-colony stimulating factor was given.

The irinotecan and the 5-FU dosages were reduced by 20% when any grade 3-4 toxicity occurred; other dose adjustments were decided on an individual basis. Treatment was stopped when the tumor progressed or severe toxicity occurred, or at the patient's request. Further treatments are discussed on an individual basis.

Assessment of therapeutic efficacy

Treatment efficacy was assessed on a clinical evaluation, carbohydrate antigen (CA) 19-9 serum levels and thoraco-abdominal computed tomography (CT). Assessment of treatment efficacy was performed every 2 mo (four cycles) or earlier in patients with clinically suspected progression. Tumor response was assessed using CT according to RECIST[23]. A complete response (CR) was defined as complete disappearance of all assessable disease, partial response (PR) as a decrease of > 30% in the sum of the largest diameters of target lesions, stable disease (SD) as a decrease of < 30% or an increase of < 20% in measurable lesions, and progressive disease (PD) as an increase of > 20% in measurable lesions or the appearance of new malignant lesions. Patients who were not assessable by CT but who presented clinical and/or biochemical (CA 19-9 serum level elevation) evidence of disease progression or who died from a cancer-related cause were also considered as PD. The sum of CR, PR and SD was reported as the tumor control rate (TCR). The sum of CR and PR was reported as overall RR (ORR). OS was defined as the time from the first day of the FOLFIRI regimen to the date of death (all causes) or last follow-up. TTP was defined as the time from the first day of the FOLFIRI regimen to the date of disease progression. Patients without progression were censored at the last follow-up.

Safety

Toxicity was assessed before each cycle with the National Cancer Institute Common Toxicity Criteria (version 3.0). A complete physical examination was performed and a full blood count and serum bilirubin, aminotransferases, alkaline phosphatase and creatinine assays were obtained before each treatment cycle.

Data collection

The following patient data were collected and analyzed retrospectively: age; gender; primary tumor location; stage at the time of diagnosis; previous surgery and/or radiotherapy; previous lines of chemotherapy; TTP with the previous line; reasons for stopping previous line; presence or absence of liver metastases at the beginning of FOLFIRI regimen; PS at the beginning of FOLFIRI regimen; type of FOLFIRI regimen (FOLFIRI-1 or FOLFIRI-3); number of cycles administered; best tumor response; grade 3-4 toxicities; dose adaptation; TTP and OS from the beginning of FOLFIRI regimen; reasons for stopping FOLFIRI regimen; further treatments.

Statistical analysis

All analyses were performed using Stata software (version 11.0; StataCorp). All statistical tests were two sided with an alpha type one error of 5%. TTP and OS were estimated using the Kaplan-Meier method and described using median or rate of TTP/OS at a specific time point with 95% CI. Log-rank tests were used to compare survival curves.

Univariate and multivariate Cox proportional hazard model analyses were performed with the following variables: stage at diagnosis; previous treatment by radiotherapy; number of previous chemotherapy lines; presence or absence of liver metastases; PS at the beginning of FOLFIRI regimen.

For exploratory purposes, subgroup analyses were performed according to the following variables: primary tumor location; stage at the diagnosis; previous treatment by surgery or radiotherapy; number of previous

Table 1 Characteristics of the 63 patients with metastatic pancreatic cancer treated with FOLFIRI after failure of gemcitabine and platinum-salts

Characteristics	Data
Age (yr)	
Median	59 (range: 24-81)
Sex (%)	
Male	33 (52.4)
Performans status (%)	
PS 0	27 (42.9)
PS 1	24 (38.1)
PS 2	12 (19.0)
Liver metastases (%)	
Present	51 (81.0)
Number of previous lines before FOLFIRI (%)	
1	19 (30.2)
2	39 (61.9)
⩾ 3	5 (7.9)

chemotherapy lines; presence or absence of liver metastases; PS at the beginning of FOLFIRI regimen; type of FOLFIRI regimen (FOLFIRI-1 or FOLFIRI-3).

RESULTS

Patients

Between January 2005 and May 2010, 63 patients with metastatic PAC fulfilled the criteria for this study. Their characteristics are shown in Table 1. Median age was 59 years (range: 24-81 years). Thirty three patients (52.4%) were male. The primary tumor was located in the head of the pancreas in 32/50 patients (64.0%). Twenty three patients (36.5%) had undergone prior surgery and 16 patients (25.4%) had received chemoradiotherapy. Twenty-seven patients (42.9%) were WHO PS 0, 24 patients (38.1%) were PS 1 and 12 patients (19.0%) were PS 2 at the beginning of the FOLFIRI regimen. Fifty one patients (81.0%) had liver metastases.

Before receiving the FOLFIRI regimen, patients had received one line (gemcitabine-oxaliplatin: n = 19, 30.2%), two lines (gemcitabine then FOLFOX regimen: n = 39, 61.9%) or three lines (n = 5, 7.9%) of chemotherapy. The previous line had been stopped for tumor progression in 55 patients (87.3%) and due to toxicity (oxaliplatin-related neuropathy) in the remaining 8 patients (12.7%).

Study treatment and drug delivery

Fifty five patients (87.3%) received the FOLFIRI-1 regimen and 8 patients (12.7%) received the FOLFIRI-3 regimen. A total of 480 cycles was completed (median: 6 cycles per patient, range: 1-51 cycles per patient).

The reasons for discontinuing the FOLFIRI regimen was progression in 56/62 patients (90.3%), toxicity in one patient (febrile neutropenia: n = 1, 1.6%), a tumor control ⩾ 6 mo or at the patient's request in 4 patients (6.5%), surgery in one patient (1.6%) who had a major response. Sixteen patients (25.4%) who remained in good condition at the time of FOLFIRI withdrawal received a subsequent line following the multidisciplinary proposal.

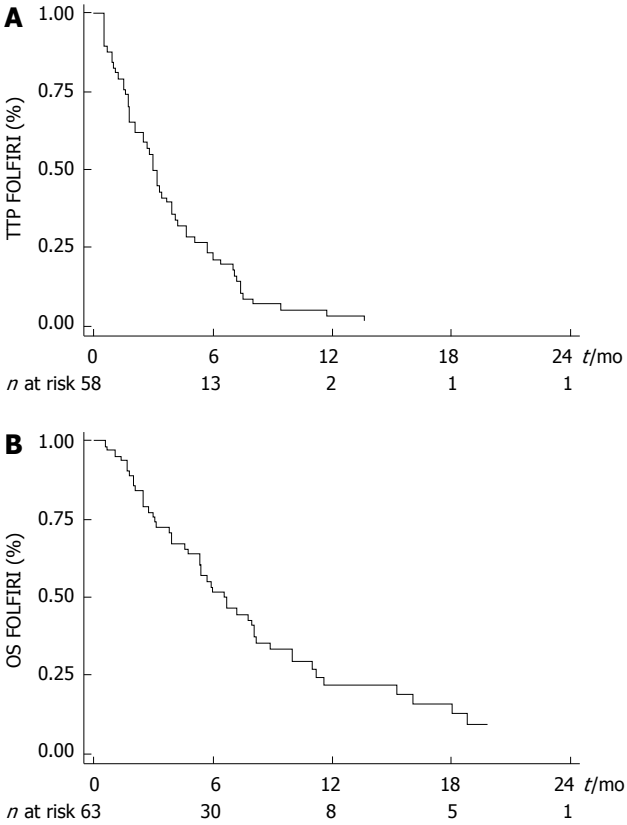


Figure 1 Time to progression (A) and overall survival (B) from the beginning of FOLFIRI for the 63 patients. TTP: Time to progression; OS: Overall survival.

Tumor response and survival

Median TTP obtained with the last line of chemotherapy before FOLFIRI was 3.9 mo (95% CI: 3.4-5.3 mo). Tumor control was obtained with the FOLFIRI regimen in 25 patients (39.7%) (CR: n = 0; PR: n = 5, 7.9%; SD: n = 20, 31.8%). ORR was 7.9% (5/63). Median TTP was 3.0 mo (95% CI: 2.1-3.9 mo) and median OS was 6.6 mo (95% CI: 5.3-8.1 mo) (Figure 1A and B).

Subgroup analysis

WHO PS was the only variable that was significantly associated with TTP and OS (Tables 2 and 3). Median TTP was 4.2 mo (95% CI: 3.2-7.0 mo) in PS 0 patients, 3.0 mo (95% CI: 1.8-4.1 mo) in PS 1 patients and 0.7 mo (95% CI: 0.5-1.5 mo) in PS 2 patients (Figure 2A). Median OS after the beginning of FOLFIRI was 8.2 mo (95% CI: 6.7-11.0 mo) in PS 0 patients, 5.4 mo (95% CI: 3.0-16.1 mo) in PS 1 patients and 2.5 mo (95% CI: 0.7-3.1 mo) in PS 2 patients (Figure 2B). PS 2 was significantly associated with a poor TTP [hazard ratio (HR) = 16.036, $P < 0.0001$] and OS (HR = 4.003, $P = 0.004$) in univariate analysis and in multivariate analysis also. No significant association was found between other variables and survival, except for the number of previous lines with TTP in multivariate analysis (Tables 2 and 3).

Dose adaptation and safety

Dose adaptation was required in 36 patients (57.1%).

Table 2 Results of univariate and multivariate analysis for time to progression with FOLFIRI

	Progression	Univariate analysis			Multivariate analysis		
	Yes/no (57/1)	HR	95% CI	*P* value	HR	95% CI	*P* value
Primary tumor location[1]				0.687			
Body or tail	18/0	1					
Head	28/1	0.883	(0.483-1.615)				
Stage at the diagnosis				0.666			0.652
Resectable	22/0	1			1		
Locally advanced	15/1	1.235	(0.631-2.418)		1.394	(0.662-2.939)	
Metastatic	20/0	0.905	(0.490-1.674)		1.299	(0.626-2.695)	
Previous surgery				0.659			
No	37/1	1					
Yes	20/0	0.882	(0.506-1.539)				
Previous radiotherapy				0.411			0.168
No	44/0	1			1		
Yes	13/1	1.305	(0.692-2.459)		1.770	(0.786-3.987)	
Number of previous chemotherapy lines				0.284			0.026
1	16/0	1			1		
2	36/1	0.649	(0.350-1.204)	0.170	0.385	(0.193-0.770)	
3	5/0	1.128	(0.411-3.096)	0.814	0.554	(0.177-1.735)	
Liver metastases				0.531			0.564
No	9/0	1			1		
Yes	48/1	0.793	(0.383-1.640)		1.298	(0.535-3.151)	
WHO performans status				< 0.0001			< 0.0001
0	24/0	1			1		
1	22/1	1.325	(0.731-2.399)		1.431	(0.745-2.748)	
2	11/0	16.036	(5.926-43.394)		29.255	(9.278-92.248)	
Type of FOLFIRI regimen				0.124			
FOLFIRI-1	50/0	1					
FOLFIRI-3	7/1	0.503	(0.210-1.207)				

HR: Hazard ratio; WHO: World Health Organization. [1]Data available in 47 out of 58 cases.

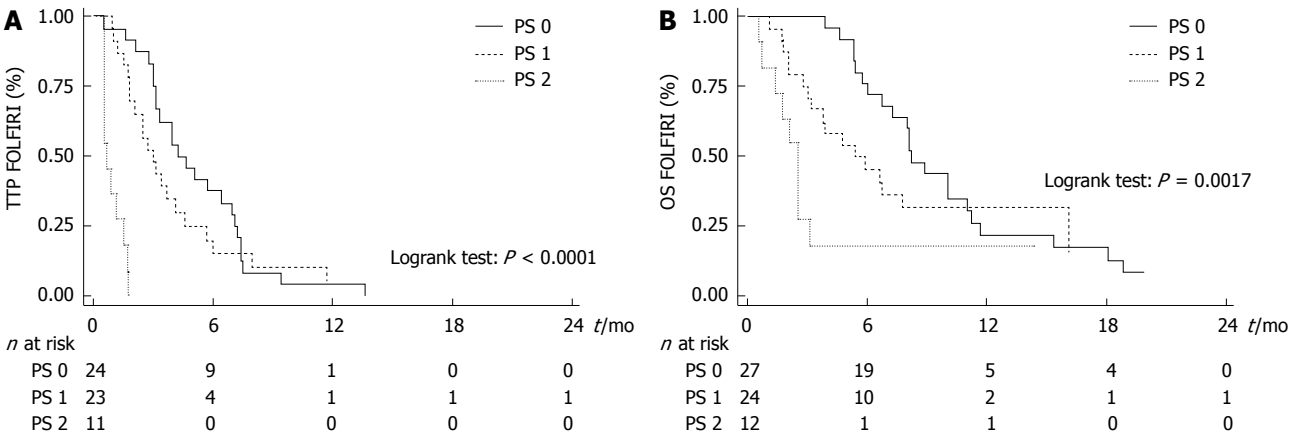


Figure 2 Subgroup analysis according to the World Health Organization performance status. A: Time to progression (TTP) according to World Health Organization (WHO) performance status (PS); B: Overall survival (OS) according to WHO PS.

The initial dose was reduced in 20 patients (31.7%) (19 patients who received the FOLFIRI-1 regimen and one patient who received the FOLFIRI-3 regimen) for the following reasons: cholestasis (n = 11), PS 2 (n = 8) or age > 75 years (n = 2), pre-existent diarrhea (n = 2) or mucositis (n = 1), and an episode of grades 3-4 hematological toxicity during previous chemotherapy (n = 1). A subsequent reduction was proposed in 19 patients (30.2%) (18 patients who received the FOLFIRI-1 regimen and one patient who received the FOLFIRI-3 regimen). Fifteen (23.8%) of these patients had grade 3-4 toxicities, mainly hematological (n = 11, 17.5%) and/or digestive with diarrhea and/or mucositis (n = 4, 6.3%). Febrile neutropenia occurred in 3 patients (4.8%). There were no related deaths.

DISCUSSION

We have evaluated the efficacy and safety of the FOLFIRI regimen after the failure of both gemcitabine and platinum salts in 63 patients with metastatic PAC treated in two centers. Tumor control was obtained in 39.7% of

Table 3 Results of univariate and multivariate analysis for overall survival with FOLFIRI

	Death	Univariate analysis			Multivariate analysis		
	Yes/no (49/14)	HR	95% CI	*P* value	HR	95% CI	*P* value
Primary tumor location[1]				0.306			
Body or tail	16/2	1					
Head	25/7	0.717	(0.380-1.355)				
Stage at the diagnosis				0.754			0.644
Resectable	20/4	1			1		
Locally advanced	13/6	1.012	(0.497-2.058)		0.790	(0.346-1.806)	
Metastatic	16/4	1.271	(0.643-2.515)		1.205	(0.577-2.514)	
Previous surgery				0.203			
No	31/9	1					
Yes	18/5	0.676	(0.370-1.236)				
Previous radiotherapy				0.916			0.935
No	38/9	1			1		
Yes	11/5	0.964	(0.491-1.894)		0.965	(0.412-2.260)	
Number of previous chemotherapy lines				0.102			0.171
1	14/5	1			1		
2	30/9	1.234	(0.649-2.346)		1.197	(0.564-2.538)	
3	5/0	3.188	(1.090-9.326)		2.945	(0.930-9.324)	
Liver metastases				0.599			0.957
No	9/3	1			1		
Yes	40/11	1.217	(0.586-2.528)		0.978	(0.444-2.159)	
WHO performance status				0.004			0.004
0	22/5	1			1		
1	18/6	1.360	(0.719-2.573)		1.284	(0.650-2.535)	
2	9/3	4.003	(1.770-9.056)		4.702	(1.883-11.741)	
Type of FOLFIRI regimen				0.856			
FOLFIRI-1	42/13	1					
FOLFIRI-3	7/1	1.083	(0.458-2.562)				

HR: Hazard ratio; WHO: World Health Organization. [1]Data available in 50 out of 63 cases.

patients. The median TTP was 3.0 mo and the median OS after the beginning of FOLFIRI was 6.6 mo. Toxicity was frequent with the FOLFIRI regimen (grade 3-4 toxicities in 23.8% of patients, mainly hematological and digestive) but manageable as only one patient had to stop treatment. In the subgroup analysis, the WHO PS was the only variable that was significantly associated with TTP (HR = 16.036, $P < 0.0001$) and OS (HR = 4.003, $P = 0.004$). Patients with WHO PS 2 may not benefit from this regimen.

Irinotecan-based chemotherapies have previously been tested in advanced PAC. Irinotecan was tested as a single agent in the first line setting in three phase Ⅱ trials with interesting results, showing an ORR of 9%-27% and a median OS of 5.2-7.3 mo[14-16]. However, two phase Ⅲ trials that tested irinotecan combined with gemcitabine as first-line chemotherapy did not show a significant benefit in TTP (2.8-3.5 mo) and OS (6.4-6.6 mo), despite a higher response rate than with standard gemcitabine (ORR: 15%-16%)[24,25]. A randomized phase Ⅱ study confirmed that the antitumoral activity of the combination of gemcitabine and irinotecan is similar to a regimen of fixed dose gemcitabine, gemcitabine/cisplatin or gemcitabine/docetaxel[26]. Thus, the gemcitabine/irinotecan combination does not seem to be synergistic. In contrast, the efficacy of the combination of irinotecan and 5-FU has been shown to be interesting with acceptable toxicity (Table 4)[20,27-29]. This is supported by *in vitro* and *in vivo* data showing synergy between these two drugs[17-19]. These regimens have not yet been tested in a phase Ⅲ trial compared with gemcitabine. Recently, a phase Ⅲ trial comparing the FOLFIRINOX regimen (folinic acid/5-FU, irinotecan and oxaliplatin combination) to gemcitabine as first-line treatment for metastatic PAC showed a significant improvement in survival with the FOLFIRINOX regimen with a median PFS and OS of 6.8 mo and 11.1 mo, respectively[7]. Toxicity was significant (grade 3-4 in 54% the patients) but manageable, and no toxic death occurred. Patients included in this study were in good condition (WHO PS 0-1). In addition, an absence of cholestasis was required for inclusion which probably explains the unusually high rate of body/tail tumor localization.

Irinotecan as a single agent has been shown to be a well-tolerated but marginally effective regimen in gemcitabine-pretreated patients. ORR was less than 10% and median OS did not exceed 4-6.6 mo[30,31]. The results with irinotecan-based combination regimens in gemcitabine-resistant advanced PAC were conflicting (Table 4)[32-37]. Data on irinotecan and 5-FU combination regimens in this setting are scarce. A randomized phase Ⅱ study evaluated modified FOLFIRI-3 *vs* modified FOLFOX (folinic acid/5-FU and oxaliplatin combination) in patients with gemcitabine-resistant advanced PAC. Efficacy was comparable with both regimens with a 6-mo OS rate of 27% (95% CI: 13%-46%) and 30% (95% CI: 15%-49%), respectively[21]. An Italian group reported a retrospective series of 40 patients with gemcitabine-

Table 4 Phase Ⅰ-Ⅱ studies of regimens as first and second lines chemotherapy in advanced pancreatic cancer

Regimen	Number of patients	TCR/ORR (%/%)	TTP or PFS (mo)	OS (mo)
Phase Ⅰ-Ⅱ studies[1]				
Irinotecan/gemcitabine/5-FU[27]	30	43/7	3.4	8.3
G-FLIP (Irinotecan/gemcitabine/5-FU/cisplatin)[28]	31	68/26	6.1	8.1
FOLFIRI-3 (Irinotecan/5-FU)[20]	40	65/38	5.6	12.1
Irinotecan/S1[29]	16	75/44	4.9	11.3
Phase Ⅱ studies[2]				
Irinotecan/raltitrexed[32]	19	53/16	4.0	6.5
IROX (Irinotecan/oxaliplatin)[33]	30	33/10	4.1	5.9
IROX (Irinotecan/oxaliplatin)[34]	14	50/21	1.4	4.1
G-FLIP (Irinotecan/gemcitabine/5-FU/cisplatin)[35]	34	44/24	3.9	10.3
Irinotecan/docetaxel[36]	14	21/0	1.2	4.4
MDI (Irinotecan/mitomycin/docetaxel)[37]	15	20/0	1.7	6.1

[1]Phases Ⅰ-Ⅱ studies of irinotecan and 5-fluorouracil (5-FU)-based regimens as first line chemotherapy; [2]Phase Ⅱ studies of irinotecan-based regimens as second line chemotherapy. TCR: Tumor control rate; ORR: Overall response rate; TTP: Time to progression; PFS: Progression free survival; OS: Overall survival.

resistant locally advanced or metastatic PAC treated with the standard FOLFIRI-1 regimen[22]. As in our series, most patients were PS 0-1 (82.5%); 17.5% of patients had locally advanced PAC, while all patients in our series were metastatic. The efficacy was quite similar to our series: TCR: 50%, ORR: 15%, median TTP: 3.7 mo and median OS: 6 mo. In contrast, toxicity was higher, with 27% and 32% of grades 3-4 hematological and digestive toxicities respectively. Toxicity was more frequent in PS 2 patients (71%) than in PS 0-1 patients (45%). The difference in the incidence of severe toxicity between the two series was not due to a difference in the proportion of PS 2 patients (17.5% *vs* 19%). One explanation might be that the initial dose was frequently adapted in our series (31.7%), particularly in PS 2 patients (8/12, 66.7%). In contrast, there was no tumor control at 6 mo in a study using a combination of irinotecan and fluoropyrimidine (mostly capecitabine) in 34 patients, most of whom where PS 0-1, and only 6% of patients were alive 1 year after the beginning of this chemotherapy regimen[38]. Most patients (97%) had been pretreated with capecitabine. This suggests that the different dose intensity and administration schedule for fluoropyrimidine, as in the XELIRI regimen, and the synergy of capecitabine and irinotecan could not overcome possible acquired resistance to this drug.

Our current study is the largest retrospective series on chemotherapy with a combination of irinotecan and 5-FU in metastatic PAC after the failure of gemcitabine and platinum salts. It was a bi-center study with a homogenous population (metastatic, not locally advanced, PAC) and selection bias was reduced by the systematic treatment policy in both centers. However, patients were heterogeneous regarding the number of previous lines of chemotherapy or the type of FOLFIRI regimen. We could not compare the efficacy of the two FOLFIRI regimens due to the unequal distribution of patients between the two groups, with only 8 patients receiving the FOLFIRI-3 regimen. Moreover, this study included selected patients treated in high volume centers with teams that were experienced in the management of pancreatic tumors and their complications. Endoscopic procedures for the treatment of jaundice, a classic exclusion criteria for irinotecan (40%-60% of PAC), were easily accessible. Another possible bias was the high rate of previous surgery (32.9%). The natural history of operated patients might be more favorable than that of patients with unresectable PAC at diagnosis. The toxicity of the FOLIFIRI regimen was manageable, although dose adaptation was required in more than half of patients (57.1%). Only patients who are PS 0-1 seem to benefit from this regimen.

In conclusion, the FOLFIRI regimen is a valuable option in patients with metastatic PAC after failure of gemcitabine and platinum salts but should be considered for patients in good condition (WHO PS 0-1). Further studies are needed to determine whether FOLFIRI is a valuable option as first line therapy in advanced PAC.

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COMMENTS

Background

Pancreatic adenocarcinoma (PAC) is the fourth leading cause of cancer death in the Western countries. The overall prognosis of metastatic PAC remains poor with a 5-year survival rate of less than 5%. Gemcitabine is the reference first-line regimen for metastatic PAC treatment. About half of patients with metastatic PAC whose disease progresses under gemcitabine are eligible for subsequent line(s) of chemotherapy and there is no standard regimen in that setting. Preclinical and clinical studies have suggested that the combination of 5-fluorouracil (5-FU) and irinotecan (FOLFIRI regimen) may be beneficial in PAC. The research aimed to evaluate the efficacy and safety of FOLFIRI regimen in patients with metastatic PAC after the failure of gemcitabine and platinum salts.

Research frontiers

Tumor response rate, toxicity of irinotecan-based regimen, time to progression and overall survival were determined for the FOLFIRI regimen.

Innovations and breakthroughs

This is a homogeneous study of consecutive metastatic PAC patients treated by two experienced teams in the management of patients with pancreatic cancer. The present paper suggests that whereas the combination of gemcitabine and irinotecan was not effective enough, that of 5-FU and irinotecan appears to be beneficial regarding both efficacy and tolerability. In addition, the study series

provides original data about the appropriate target population for the irinotecan-based regimen, particularly taking into account the PS status.

Applications

Recently, the FOLFIRINOX schema, combining 5-FU, irinotecan and oxaliplatin, was shown to be superior to gemcitabine in a first-line setting, with an overall survival of 11.1 mo (95% CI: 9-13.1 mo) *vs* 6.8 mo (95% CI: 5.5-7.6 mo, hazard ratio = 0.57) ($P < 0.0001$), respectively, in selected patients (performance status 0-1, absence of cholestasis). However, due to the hematological toxicity of this combination, many patients are not eligible for first-line therapy. The sequence FOLFOX then FOLFIRI (or the reverse) may be an alternative and should be considered as being better tolerated.

Terminology

For treatment purposes, pancreatic tumors are generally classified as resectable, locally advanced, or metastatic. A locally advanced pancreatic cancer is a tumor involving the arterial axis (celiac trunk, mesenteric artery) and thus is not resectable despite no detectable metastases. This form of cancer should be distinguished from metastatic tumors as the prognosis is different (slightly better, and some patients can have surgical treatment in case of a good tumor response after chemotherapy) and separate analyses are needed. Thus, locally advanced PAC patients were excluded from the study.

Peer review

This is an interesting study in which authors evaluate the efficacy and safety of this regimen in patients with metastatic PAC after the failure of gemcitabine and platinum salts. The results are convincing and suggest that FOLFIRI regimen had an acceptable toxicity and an interesting efficacy in our study, limited to patients in good condition.

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BRIEF ARTICLE

Efficacy of a therapeutic strategy for eradication of *Helicobacter pylori* infection

Giuliana Sereni, Francesco Azzolini, Lorenzo Camellini, Debora Formisano, Francesco Decembrino, Veronica Iori, Cristiana Tioli, Maurizio Cavina, Francesco Di Mario, Giuliano Bedogni, Romano Sassatelli

Giuliana Sereni, Francesco Azzolini, Lorenzo Camellini, Francesco Decembrino, Veronica Iori, Cristiana Tioli, Maurizio Cavina, Giuliano Bedogni, Romano Sassatelli, Unit of Gastroenterology and Digestive Endoscopy, Arcispedale Santa Maria Nuova, Istituto di Ricovero e Cura a Carattere Scientifico, 42123 Reggio Emilia, Italy
Debora Formisano, Statistics and Clinical Epidemiology Unit, Arcispedale Santa Maria Nuova, Istituto di Ricovero e Cura a Carattere Scientifico, 42123 Reggio Emilia, Italy
Francesco Di Mario, Unit of Gastroenterology, Ospedale S. Maria di Ca' Foncello, 31100 Treviso, Italy
Author contributions: Sereni G, Azzolini F, Camellini L, Decembrino F, Iori V, Tioli C, Cavina M and Sassatelli R treated and included the patients in the study and had access to the data; Azzolini F generated a computer-based database of all patients; Sereni G planned the study, organized and analyzed the data, discussed with all the authors the statistical analysis report and wrote the manuscript; Formisano D contributed to the statistical analysis of the data; Camellini L, Sassatelli R, Bedogni G and Di Mario F participated in the editing of the final report.
Correspondence to: Giuliana Sereni, MD, PhD, Unit of Gastroenterology and Digestive Endoscopy, Arcispedale Santa Maria Nuova, Istituto di Ricovero e Cura a Carattere Scientifico, Viale Risorgimento 80, 42123 Reggio Emilia, Italy. giuliana.sereni@asmn.re.it
Telephone: +39-522-296947 Fax: +39-522-295941
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Abstract

AIM: To determine the efficacy of our therapeutic strategy for *Helicobacter pylori* (*H. pylori*) eradication and to identify predictive factors for successful eradication.

METHODS: From April 2006 to June 2010, we retrospectively assessed 2428 consecutive patients (1025 men, 1403 women; mean age 55 years, age range 18-92 years) with gastric histology positive for *H. pylori* infection referred to our unit for 13-C urea breath test (UBT), after first-line therapy with proton pump inhibitor (PPI) *b.i.d.* + amoxicillin 1 g *b.i.d.* + clarithromycin 500 mg *b.i.d.* for 7 d. Patients who were still positive to UBT were recommended a second-line therapy (PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + tinidazole 500 mg *b.i.d.* for 14 d). Third choice treatment was empirical with PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + levofloxacin 250 mg *b.i.d.* for 14 d.

RESULTS: Out of 614 patients, still *H. pylori*-positive after first-line therapy, only 326 and 19 patients respectively rechecked their *H. pylori* status by UBT after the suggested second and third-line regimens. "Per protocol" eradication rates for first, second and third-line therapy were 74.7% (95% CI: 72.7%-76.4%), 85.3% (95% CI: 81.1%-89.1%) and 89.5% (95% CI: 74.9%-103%) respectively. The overall percentage of patients with *H. pylori* eradicated after two treatments was 97.8% (95% CI: 97.1%-98.4%), *vs* 99.9% (95% CI: 99.8%-100%) after three treatments. The study found that eradication therapy was most effective in patients with ulcer disease ($P < 0.05$, $P = 0.028$), especially in those with duodenal ulcer. Smoking habits did not significantly affect the eradication rate.

CONCLUSION: First-line therapy with amoxicillin and clarithromycin produces an *H. pylori* eradication rate comparable or superior to other studies and second-line treatment can still be triple therapy with amoxicillin and tinidazole.

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Key words: *Helicobacter pylori*; Eradication treatment; Rescue therapy; Eradication rate; Triple therapy; First-line therapy; Second-line therapy

Peer reviewers: Dr. David J McGee, Microbiology and Immunology, Louisiana State University Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130, United States; Dr. Tamara Vorobjova, Department of Immunology,

University of Tartu, Ravila 19, 50411 Tartu, Estonia

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a bacterium that colonizes the stomach of about half the world's population and is considered the main cause of peptic ulcer[1,2] and mucosa-associated lymphoid tissue lymphoma[3,4]; furthermore it is an important risk factor for gastric adenocarcinoma[5,6] and also an associated factor for many gastric and non-gastric diseases[7]. *H. pylori* eradication is strongly recommended[8,9] in many clinical situations[10]. Different treatments have been proposed as effective[11,12] and meta-analyses are available comparing their results[13-15]. Prevalence of antibiotic resistance was found to be an important factor in the choice of the preferred regimen[16-19].

In particular, clarithromycin resistance is the strongest predictor of treatment failure[20,21]: in fact the eradication rate decreases below the recommended 80% threshold, when the prevalence of clarithromycin resistance reaches 15%-20%. On the other hand *in vitro* resistance to metronidazole may not accurately reflect resistance *in vivo*, and does not significantly affect the eradication rate[22]. In Northern Italy primary resistance to clarithromycin and metronidazole is reported to be 16.6% and 25.1% respectively[23].

The American College of Gastroenterology guidelines recommend first-line eradication with triple therapy consisting of a proton pump inhibitor (PPI), amoxicillin and clarithromycin. The European (Maastricht Ⅲ Consensus) guidelines recommend first-line eradication with triple therapy on account of clarithromycin/metronidazole resistance. According to both guidelines, second-line therapy should be a bismuth-based quadruple treatment while third-line treatment should be based on antimicrobial susceptibility testing[24].

Our Trust policy is to suggest an antibiotic regimen to outpatients referred to our unit for 13-C urea breath test (UBT), or esophagogastroduodenoscopy (EGD) with assessment of the *H. pylori* status and found to be *H. pylori*-positive. We do not usually prescribe a bismuth-based second-line therapy and we do not base the choice of the third-line therapy on antimicrobial susceptibility testing. The aim of the present study is to determine the efficacy of this strategy for *H. pylori* eradication.

MATERIALS AND METHODS

Setting

Our UBT outpatient clinic is the only existing one in the Health District of Reggio Emilia, serving about 500 000 people in North Italy. EGD is performed in six different public hospitals and in several private practices.

Patients

Our policy was to suggest an eradication therapy to the general practitioners of outpatients found to be *H. pylori*-positive after EGD or 13-C UBT performed at our Unit. Before examination (EGD or UBT), a brief medical history was obtained, including reason for investigation, allergy, past and ongoing treatments, and smoking habits. In patients undergoing EGD, if *H. pylori* status assessment was indicated, it was obtained by histology, according to the Sydney classification.

UBT was performed using an isotope ratio mass spectrometer (Breathmat, Bremen). A delta value > 3.5 over baseline was considered a positive result. All patients *H. pylori*-positive after EGD or UBT received a letter for their general practitioner reporting the result of the test, the recommended antibiotic regimen and the suggestion to further submit the patient to our UBT outpatient clinic to confirm *H. pylori* eradication, at least 8 wk after the end of treatment. Standard first-, second- and third-line antibiotic regimens are shown in Table 1. Alternative antibiotic regimens were suggested to patients with known allergy to a particular antibiotic. Each patient also received a form, where she/he was instructed to mark consumption of the drugs and to record any suspected adverse effect. Compliance with the proposed treatment and adverse effects were checked, on the occasion of the resubmission of the patient. Nevertheless no attempt was made to contact patients, who did not return to our UBT outpatient clinic and no information about them was actively sought. Data about patients and treatments were prospectively collected in a purpose-built database.

In the present observational retrospective study, we included 2428 outpatients (Table 2 shows their characteristics), who underwent UBT from April 2006 to June 2010, after positive histological assessment of *H. pylori* status and after first-line eradication therapy with PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + clarithromycin 500 mg *b.i.d.* for 7 d.

Ethics and endpoints

The study was conducted according to the declaration of Helsinki. All patients consented to UBT. Although patients were informed about the purpose and potential side effects of the suggested treatments, a formal consensus about therapy was not obtained, because the therapy was always finally prescribed by the general practitioner after further evaluation and discussion with the patient. Furthermore none of the medications were prescribed as part of a clinical trial and therefore there was no need for study approval by the ethics committee at our hospital. The primary endpoints of the study were eradication rates of the suggested antibiotic regimens, as calculated per protocol in patients, who were referred to our UBT outpatient clinic after each line of treatment. Secondary endpoints were: (1) percentage of patients, still *H. pylori*-positive after first-line therapy, who achieved

Table 1 Antibiotic regimens suggested by our unit and eradication rates per protocol

	Patients	*H. pylori* eradicated and eradication rate per protocol (95% CI)	Patients treated and cumulative effect (95% CI)
First-line: PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + clarithromycin 500 mg *b.i.d.* for 7 d	2428	1814 74.7 (72.7-76.4)	
Second-line: PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + tinidazole 500 mg *b.i.d.* for 14 d	326	278 85.3 (81.1-89.1)	2140 97.8 (97.1-98.4)
Third-line: PPI *b.i.d.* + amoxicillin 1 g *b.i.d.* + levofloxacin 250 mg *b.i.d.* for 14 d	19	17 89.5 (74.9-103)	2111 99.9 (99.8-100)

PPI: Proton pump inhibitor; b.i.d.: Twice daily; *H. pylori*: *Helicobacter pylori*. Standard dosages for PPIs are as follows: lansoprazole 30 mg, omeprazole 20 mg, pantoprazole 40 mg, rabeprazole 20 mg, esomeprazole 20 mg.

Table 2 Characteristics of patients who underwent urea breath test after treatment (%)

Patients characteristics	UBT after first-line therapy (2428 patients)	UBT after second-line therapy (326 patients)	UBT after third-line therapy (19 patients)
Women	1403 (58)	202 (62)	13 (68)
Men	1025 (42)	124 (38)	6 (32)
Mean age (range), yr	55 (18-92)	54 (21-86)	53 (27-80)
Peptic ulcer disease	202 (8.2)[1]	23 (7.1)	0
Previous gastric surgery and/or neoplasm	6 (0.2)[2]	3 (0.9)	0
Smokers	424/1082 (43.8)	59/287 (20.6)	3/17 (7.6)

[1]172 and 23 patients were found to be affected respectively by duodenal and gastric ulcer. 7 patients were affected by both gastric and duodenal ulcer; [2]1 patient was affected by low-grade mucosa-associated lymphoid tissue. Among the patients with a history of previous gastric surgery, 1 patient had undergone surgery for cancer, the others for peptic ulcer disease. UBT: Urea breath test.

H. pylori eradication, proved by UBT, at the end of study; and (2) factors predictive of successful eradication.

Statistical analysis

The data was analysed with SPSS v.18.0 (Chicago, Illinois United States). The significance level is 0.05. The data is summarized by the primary measures of central tendency and dispersion, as well as with frequencies and percentages with 95% CI. Pearson's Chi square and Fisher's exact test were used to compare frequencies, where appropriate.

RESULTS

The study flow chart is shown in Figure 1. Six hundred and fourteen patients (25.3%) were found to be *H. pylori*-positive to UBT, after the first-line antibiotic regimen.

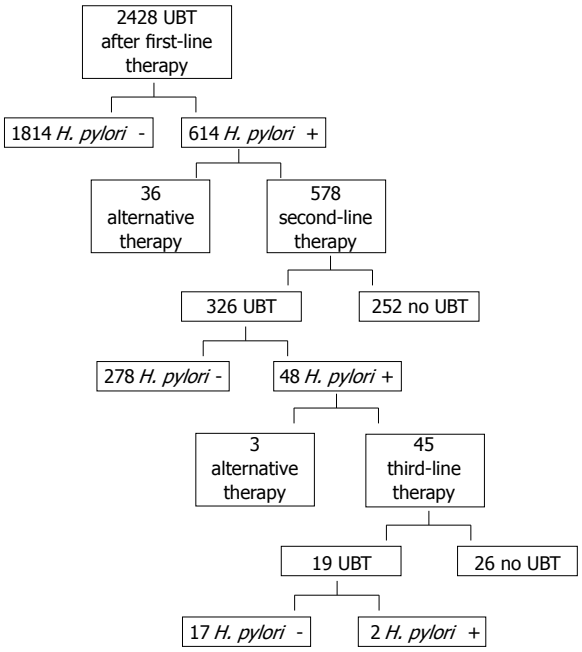


Figure 1 Flow chart of the study. UBT: Urea breath test; *H. pylori*: *Helicobacter pylori*.

Out of these patients, 326 (53.1%) were referred again to our unit to check eradication after second-line standard therapy. Out of the 48 still *H. pylori*-positive after second-line therapy, only 19 underwent UBT (39.6%).

An antibiotic regimen alternative to the second or third-line standard therapy was suggested by us, or by their general practitioner, to 40 patients (6.5%). Among the patients, who were referred to us for UBT, a small number reported a non-complete compliance with the received standard antibiotic regimen, because of side effects: this was the case in 13 patients (0.5%, 11 tested *H. pylori*-negative and 2 positive) during first-line therapy and in 9 patients (2.7%, all eradicated) during second-line therapy. Three out of 21 reported a previously unknown allergy to penicillin; all other side effects were minor (diarrhea, headache, metallic taste).

Eradication rates of the antibiotic regimen, as calculated in patients who subsequently checked *H. pylori* status by UBT, are reported in Table 1. The two patients who were still positive after the third treatment were advised to take bismuth-based quadruple therapy for 14 d and *H. pylori* was finally eradicated.

As shown in Table 3, peptic ulcer disease, but not smoking habits, was found to be predictive of successful eradication during first-line therapy. Stratifying patients according to the number of cigarettes smoked per day (data not shown), the eradication rate decreased proportionally to the increase in the number of cigarettes, though statistical significance was only borderline ($P = 0.056$).

Table 4 compares that characteristics of the patients who rechecked (297, 48.4%) or not (278) their *H. pylori* status, until the achievement of an UBT-proved *H. pylori*-negative status, or after the completion of third-line therapy, whichever came first.

Table 3 Factors investigated as potentially predictive of successful eradication (%)

Risk factor	Eradication rate	
	First-line antibiotic regimen	Second-line antibiotic regimen
Women	1029/1403 (73.3)	172/202 (85.1)
Men	785/1025 (76.6)	106/124 (85.5)
Peptic ulcer disease	167/206 (81.1)[1]	22/24 (91.7)
Non-ulcer disease	1647/2222 (74.1)[1]	54/71 (76.1)
Smokers	302/424 (71.2)	50/57 (94.7)
Non-smokers	1346/1 778 (75.7)	192/230 (83.5)

[1]Difference of frequencies: 6.9% (95% CI: 1%-12.8%, $P < 0.05$). If patients affected by duodenal ulcers is compared with patients with non-ulcer disease, the difference is 8.3% (95% CI: 2.3%-14.4%, $P < 0.05$).

Table 4 Characteristics of patients who rechecked or not their *Helicobacter pylori* status (%)

Patients characteristics	Rechecked their *H. pylori* status[1] (297 patients)	No recheck of their *H. pylori* status (278 patients)
Women/men	185 (62)/112 (38)	170 (61)/108 (39)
Mean age (range), yr	55 (21-86)	50 (20-92)
Peptic ulcer disease	21 (7)	17 (6)
Previous gastric surgery and/or neoplasm	2 (0.6)	1 (0.3)
Smokers	53 (20)	69 (27)

[1]Patient who rechecked their *Helicobacter pylori* (*H. pylori*) status, until the achievement of an urea breath test-proved *H. pylori*-status, or after the completion of the third-line therapy, whichever came first. Patients treated with alternative second and third-line antibiotic regimens are excluded.

DISCUSSION

In our study, patients were treated with first-line PPI-amoxicillin-clarithromycin therapy for 7 d only. A recent meta-analysis showed a minimal advantage of longer treatment[25].

All the different PPIs (omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole) are shown to be equally effective in combination with antibiotic therapy[26] and higher H2 receptor antagonists (ranitidine)[27]: therefore we left the choice of PPI to the general practitioner. We also advised general practitioners to prescribe the double dose of PPI, because it has proved more effective in a recent meta-analysis[28].

The eradication rate after first therapy was 74.7%. This rate is comparable to the 76% achieved by the same regimen (for 10 d of treatment) in a recent study from Greece[29] and it is higher than the 56% found in a study with 14 d of PPI-clarithromycin-metronidazole[30,31].

The European and American guidelines recommend a bismuth-based quadruple treatment as second-line therapy; according to Maastricht guidelines, a PPI-amoxicillin-metronidazole therapy could be used if bismuth is not available.

In Italy bismuth salts are often available, but we preferred to recommend PPI-amoxicillin-tinidazole as second-line therapy, because the bismuth-based quadruple regimen is associated with a relatively high incidence of side effects and a worse compliance than the triple regimen; although a recent meta-analysis shows no statistically significant differences[32]. The PPI-amoxicillin-metronidazole regimen was reported to be effective as second-line therapy to achieve eradication rates of 89% and 64% for metronidazole susceptible and resistant strains, respectively[33]. In our study, this second-line regimen obtained an eradication rate of 85.3%. These results are superior to data reported by others after unsuccessful first-line treatment for the same regimen, for the bismuth-based quadruple combination[34] and also for a levofloxacin-based therapy[35].

The Consensus Conference recommended third-line rescue treatment based on antimicrobial susceptibility testing. We preferred not to perform susceptibility testing, choosing an empirical triple therapy with levofloxacin.

Two meta-analyses have suggested that levofloxacin-based rescue regimen is more effective and safer than quadruple therapy[35,36]. Levofloxacin resistance develops rapidly and it is one of the most effective antibiotics in the treatment of respiratory tract infections. For these reasons, we reserved its use for third-line therapy. In our study, levofloxacin-based third-line therapy achieved an eradication rate of 89.5%. Although we observed these excellent results in a small group of patients, our findings are consistent with other experiences[29,36] and support the use of a levofloxacin-based regimen as empirical third-line therapy of *H. pylori* infection.

The overall eradication rate after two and three treatments, respectively, is proved to be 97.8% and 99.9%. These results confirm the data already published in literature (Table 5).

During first-line treatment, the efficacy of therapy in patients with peptic ulcer disease was higher (81.1%) than in patients with non-ulcer disease (74.1%). This finding is in agreement with others experiences[37-39] and could be explained by a larger number of antibiotic-resistant *H. pylori* strains in non-ulcer disease patients.

The study also assessed the influence of smoking on treatment: the eradication rate was moderately higher in non-smokers (75.7% *vs* 71.2%). Recent studies demonstrated that smoking affects the eradication rate[40,41]; in our larger report the trend is negative but not yet statistically significant.

It should be noted that our study is not a clinical trial, but a retrospective revision of our practice. This may explain its major limitation; that is the high number of patients (45.3%), who did not have their *H. pylori* status rechecked after failure of first-line therapy. We do not know whether these patients did not undergo any further treatment, or they stopped therapy because of adverse effects, or decided not to repeat UBT after completion of the suggested regimen. Nevertheless the regimens suggested in our studies are usually relatively well tolerated and we do not believe that the high percentage of our patients lost at follow up was due to adverse effects. In the setting of an open-access UBT facility,

Table 5 Studies reporting eradication rates and cumulative effect of different therapeutic strategies

	% Eradication rate (number of patients)				
	First-line	Second-line	Third-line	Cumulative effect (Ⅱ to Ⅲ line)	Antimicrobial susceptibility testing before third-line
Gasbarrini *et al*[41] 2000	86 (2413)	82 (329)	77 (39)	99	Yes
Beales[42] 2001	73 (469)	70-73 (66)	65 (20)	94-98	Yes
Qasim *et al*[40] 2005	77 (3280)	56 (270)	38 (28)		
Gisbert *et al*[32] 2008	70 (500)	74 (343)	76 (136)	99.5	No
Rokkas *et al*[28] 2009	76 (540)	73 (120)	70 (30)	98	No
Present study	75 (2428)[1,2]	85 (326)	89 (19)	97.8-99.9	No

[1]172 and 23 patients were found to be affected respectively by duodenal and gastric ulcer. 7 patients were affected by both gastric and duodenal ulcer; [2]1 patient was affected by low-grade mucosa-associated lymphoid tissue. Among the patients with a history of previous gastric surgery, 1 patient had undergone surgery for cancer, the others for peptic ulcer disease. UBT: Urea breath test.

Qasim *et al*[40] reported that only 25% of their patients *H. pylori*-positive after first-line therapy, were proved to have eradication of *H. pylori* after second-line therapy or have rechecked their *H. pylori* status by UBT after third-line therapy. A high compliance with repeated courses of therapy is reported in prospective clinical trials[29,41], but it is conceivable that it may be difficult to replicate these good results in common clinical practice. Data are lacking about strategies useful to reinforce compliance with *H. pylori* eradication therapy in this setting. It should be noted that a high percentage of our patients did not achieve a proved *H. pylori*-negative status, despite the fact that our policy avoided invasive testing (EGD) to obtain antimicrobial susceptibility testing and regimens requiring assumption of a lot of pills, such as bismuth-based quadruple therapy.

We believe that efforts to introduce new effective antibiotic regimens, should parallel not only the emergence of new resistance, but also the efforts to target appropriate antibiotic regimens to the majority of patients requiring *H. pylori* eradication therapy. Our policy to suggest to the general practitioner an antibiotic regimen for each patient found to be *H. pylori*-positive may be criticized and it could be not generally applicable. Nevertheless different models of collaboration between gastroenterologists and general practitioners should be tested to improve effectiveness of *H. pylori* infection therapy in current practice.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) is considered an important risk factor for many gastric and non-gastric diseases. Different treatments have been proposed as effective for *H. pylori* eradication and meta-analyses are available comparing their results. Prevalence of antibiotic resistance was found to be an important factor in the choice of the preferred regimen.

Research frontiers

The European (Maastricht Ⅲ Consensus) and American (American College of Gastroenterology) guidelines recommend a therapeutic strategy for *H. pylori* eradication with clarithromycin and metronidazole for 7-14 d or amoxicillin and clarithromycin for 10-14 d as first-line therapy. They also suggest bismuth-based quadruple second-line therapy and third-line therapy based on antimicrobial susceptibility tests.

Innovations and breakthroughs

In the present study, about 75% of patients were cured with first-line therapy (proton pump inhibitor + amoxicillin + clarithromycin), 97.8% after the second-line therapy (proton pump inhibitor + amoxicillin + tinidazole) and 99.9% after the third-line therapy (proton pump inhibitor + amoxicillin + levofloxacin). No statistical differences in eradication rates in smokers and non-smokers were found, even if there is a negative trend. Patients with ulcer disease had an eradication rate superior to that of patients with non-ulcer disease. This study was conducted in an open access setting and showed only about half of the patients still *H. pylori*-positive after first-line therapy were finally proved to have successful eradication of *H. pylori*.

Applications

The high eradication rates of this study suggest that a regimen simpler than quadruple drug therapy may be preferred as second-line therapy at least in some patients and in some regions; furthermore the antimicrobial susceptibility test (and the esophagogastroduodenoscopy involved) would not be necessary after two failed treatments. The fact that nearly half of our patients still *H. pylori*-positive after first-line therapy did not reach a urea breath test (UBT)-proved *H. pylori*-negative status, despite the use of effective antibiotic regimens, suggests that strategies should be devised and implemented to improve the access of patients to appropriate therapies and to strengthen the compliance of patients to these treatments.

Terminology

H. pylori is a bacterium that colonizes the stomach of about half the world's population and is considered the main cause of peptic ulcer and mucosa-associated lymphoid tissue lymphoma; furthermore it is an important risk factor for gastric adenocarcinoma and also an associated factor for many gastric and non-gastric diseases. *H. pylori* infection can be detected by esophagogastroduodenoscopy (a biopsy is taken during endoscopy and it is sent to the hospital laboratory to be examined for histology) or UBT. In the 13-C UBT, person fast for about 6 h. A baseline breath sample is collected (person blow into a test tube), then person drink a solution of Carbon-13-urea in water. A second breath sample is taken after 30 min and analyzed by a mass spectrometer. If *H. pylori* is present in the stomach the Carbon-13-urea will be broken down and Carbon-13 will appear in the breath.

Peer review

These authors conducted a retrospective epidemiology study on over 2428 patients who were positive for *H. pylori* looking to determine the efficacy of a therapeutic strategy for *H. pylori* eradication based on first-line therapy with amoxicillin and clarithromycin and second-line treatment with amoxicillin and tinidazole. The authors found that the eradication therapy was most effective in patients with ulcer disease, especially with duodenal ulcer during first-line therapy.

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BRIEF ARTICLE

Lack of CD44 variant 6 expression in rectal cancer invasive front associates with early recurrence

Suvi Tuulia Avoranta, Eija Annika Korkeila, Kari Juhani Syrjänen, Seppo Olavi Pyrhönen, Jari Toivo Tapio Sundström

Suvi Tuulia Avoranta, Eija Annika Korkeila, Kari Juhani Syrjänen, Seppo Olavi Pyrhönen, Department of Oncology and Radiotherapy, University of Turku and Turku University Hospital, FIN-20521 Turku, Finland
Jari Toivo Tapio Sundström, Department of Pathology, University of Turku and Turku University Hospital, FIN-20520 Turku, Finland
Author contributions: All authors designed the research; Avoranta ST and Korkeila EA collected the clinicopathological data; Avoranta ST and Sundström JTT analyzed the immunohistochemical staining; Avoranta ST and Syrjänen KJ performed the statistical work; Avoranta ST wrote the paper; Korkeila EA, Syrjänen KJ, Pyrhönen SO and Sundström JTT gave comments and suggested revisions.
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Correspondence to: Tuulia Avoranta, MD, Department of Oncology and Radiotherapy, University of Turku and Turku University Hospital, Hämeentie 11, FIN-20521 Turku, Finland. stavor@utu.fi
Telephone: +35-82-3131834 Fax: +35-82-3132809
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Abstract

AIM: To investigate the prognostic value of CD44 variant 6 (CD44v6), a membranous adhesion molecule, in rectal cancer.

METHODS: Altogether, 210 rectal cancer samples from 214 patients treated with short-course radiotherapy (RT, *n* = 90), long-course (chemo) RT (*n* = 53) or surgery alone (*n* = 71) were studied with immunohistochemistry for CD44v6. The extent and intensity of membranous and cytoplasmic CD44v6 staining, and the intratumoral membranous staining pattern, were analyzed.

RESULTS: Membranous CD44v6 expression was seen in 84% and cytoplasmic expression in 81% of the cases. In 59% of the tumors with membranous CD44v6 expression, the staining pattern in the invasive front was determined as "front-positive" and in 41% as "front-negative". The latter pattern was associated with narrower circumferential margin (P = 0.01), infiltrative growth pattern (P < 0.001), and shorter disease-free survival in univariate survival analysis (P = 0.022) when compared to the "front-positive" tumors.

CONCLUSION: The lack of membranous CD44v6 in the rectal cancer invasive front could be used as a method to identify patients at increased risk for recurrent disease.

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Key words: CD44 variant 6; Rectal cancer; Invasive front; Disease-free survival; Disease-specific survival

Peer reviewer: Dr. Cem Onal, Department of Radiation Oncology, Baskent University Faculty of Medicine, Adana Research and Treatment Center, 01120 Adana, Turkey

Avoranta ST, Korkeila EA, Syrjänen KJ, Pyrhönen SO, Sundström JTT. Lack of CD44 variant 6 expression in rectal cancer invasive front associates with early recurrence. *World J Gastroenterol* 2012; 18(33): 4549-4556 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i33/4549.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i33.4549

INTRODUCTION

In 2008, approximately 1.2 million new cases of colorectal cancer (CRC) were diagnosed globally[1]. Preoperative radiotherapy (RT) for rectal cancer has improved local control rates[2], but patients may still present with fairly differing clinical responses and prognoses[3]. To improve

disease predictability, strong expectations have been placed on tumor markers.

CD44 is a family of transmembrane glycoproteins serving as a major receptor for hyaluronate, an important component of the extracellular matrix (ECM)[4]. *CD44* has been suggested to act both as a tumor-suppressing cofactor and as a growth- and invasiveness-promoting molecule[5] through its participation in many important cellular processes, including adhesion, growth regulation, survival, differentiation and motility[6]. Of the several isoforms produced by alternative splicing of the CD44 gene[7], variant 6 (CD44v6) has been intensely studied in relation to CRC progression and outcome[8].

Induction in CD44v6 expression is suggested to represent an early event in colorectal carcinogenesis[9], and has in some studies been related to disease progression, metastatic potential[7,9,10], and poor disease outcome[11]. Instead, in some other studies, stronger CD44v6 expression has been reported in adenomas than in carcinomas, as well as in primary carcinomas compared to metastatic tumors[12], and has been shown to have favorable[8,13] or no[14] effect on CRC outcome. Furthermore, strong expression has been shown to indicate more favorable response to chemotherapy[15].

In most of the previous studies on CD44v6 expression in colorectal tumors, both colonic and rectal carcinomas have been included. There is, however, some evidence that proximal and distal colonic lesions differ in their expression of CD44v6[16]. In those few studies including solely rectal tumors[17-19], CD44v6 has not been considered in relation to RT. In the present study, we examined the expression of CD44v6 immunohistochemically in a cohort of 214 primary rectal carcinomas treated with or without preoperative (chemo)radiotherapy. Considering the fundamental role of the tumor invasive front in tumor-host interaction[20], as well as the discrepant data on the prognostic value of the extent of CD44v6 expression in CRC[8,11,14], intratumoral staining pattern of this protein was also systematically assessed. We hypothesized that this aspect could offer additional information of the significance of CD44v6 expression in rectal cancer.

MATERIALS AND METHODS

Patients and study material

The material of this study consisted of formalin-fixed, paraffin-embedded tissue samples from 214 patients operated upon for rectal cancer at Turku University Hospital between 2000 and 2009. Operative samples were retrieved from the archives of the Department of Pathology, Turku University Hospital. To ensure a biologically and therapeutically homogeneous study population, only tumors of the middle and lower rectum were included. Superficial tumors treated with excision only, as well as patients with distant metastases at the time of diagnosis, were excluded. The use of archival tissue material was approved by the National Supervisory Authority for Welfare and Health (permission No. Dnro 1709/32/300/02, May 13th 2002).

Tumor staging was done according to the tumor node metastasis classification of malignant tumors, 2002[21]. Selection of treatment was based on preoperative tumor staging which included computed tomography (CT) or magnetic resonance imaging of the rectum, CT of the abdomen, and X-ray or CT of the chest. According to the common clinical guidelines[22], patients were treated either with short-course preoperative RT (n = 90), long-course preoperative (chemo) RT (n = 53), or received no treatment before surgery (n = 71). Short-course RT consisted of five 5-Gy fractions during 1 wk, with surgery on the following week. Long-course RT was given in 1.8-Gy fractions to a total dose of 50.4 Gy over a 6-wk period, with (n = 44) or without (n = 9) concomitant chemotherapy, and operation was performed at 5-7 wk after RT. Chemotherapy regimens were either bolus 5-fluorouracil (n = 5) or capecitabine (n = 39). Anterior resection was performed in 118 cases (55%), and abdominoperineal resection in 92 cases (43%). In four cases (2%), some other technique, such as low Hartmann's procedure, was used. The presence of vascular invasion was assessed in 159 cases, in which cancer cells could be detected in 47 cases (30%), either in the extramural lymphatic or blood vessels. Postoperative adjuvant chemotherapy was given to patients with lymph node positive or high-risk lymph node negative tumors according to the standard clinical practice[22]. The median follow-up time was 45.5 mo. In 61 patients (29%), local or distant disease recurrence was seen. Of them, 39 (64%) were treated with chemotherapy with or without biological treatments. Among these 39 cases, progression-free survival (PFS) was retrospectively defined from the medical records, and the median PFS was 20.7 mo. The key demographic and clinical characteristics of the patients are summarized in Table 1.

Immunohistochemistry

Four tumors with T0 after long-course RT were not stained, and accordingly, the actual study material consisted of 210 samples. The most representative blocks were selected and cut into 5-μm sections. Antigen retrieval was performed by heating in a microwave oven in 10 mmol/L sodium citrate, pH 6.0 two times for 7 min. Endogenous peroxidase activity was blocked by incubating the slides in 0.3% hydrogen peroxide in Tris-buffered saline. The sections were subjected to immunohistochemical staining with monoclonal antibody for CD44v6 (concentration 1:1000; Bender MedSystems, Vienna, Austria) and monoclonal mouse anti-cytokeratin (Ck-Pan) (clone: AE1/AE3, concentration 1:50; Zymed Laboratories, South San Francisco, CA, United States) using EnVision + Dual Link System - HRP (Dako, Denmark).

Evaluation of immunohistochemistry for CD44v6

Immunohistochemistry (IHC) staining was individually evaluated by two observers (Avoranta ST and Sundström

Table 1 Clinical characteristics of the patients *n* (%)

Clinical characteristics	Short course RT	Long course RT	Control	P^1
Study population (n = 214)				
Male	57 (63)	34 (64)	32 (45)	0.03
Female	33 (37)	19 (36)	39 (55)	
Mean age (yr)	65.2	64.7	74.5	< 0.001
Preoperative T				
T1-2	28 (31)	0 (0)	22 (31)	
T3	54 (60)	2 (4)	12 (17)	< 0.001
[2]T4	1 (1)	50 (94)	3 (4)	
Tx	7 (8)	1 (2)	34 (48)	
Postoperative T				
T1	3 (3)	2 (4)	5 (7)	
T2	34 (38)	6 (11)	26 (37)	
T3	50 (56)	27 (51)	37 (52)	< 0.001
T4	3 (3)	14 (26)	3 (4)	
T0	0	4 (8)	0	
Postoperative N				
N0	53 (59)	36 (68)	41 (58)	
N1	26 (29)	14 (26)	15 (21)	0.33
N2	11 (12)	3 (6)	12 (17)	
Nx	0	0	3 (4)	
Grade				
G1	9 (10)	10 (19)	12 (17)	
G2	58 (64)	33 (62)	48 (68)	0.07
G3	21 (23)	3 (6)	11 (16)	
Gx	2 (2)	7 (13)	0	
[3]Crm (mm)				
0	3 (4)	11 (25)	4 (9)	
0 ⩽ crm ⩽ 2	9 (12)	7 (16)	7 (16)	0.009
> 2	65 (84)	26 (59)	32 (74)	
Disease specific outcome				
Alive without recurrence	64 (71)	25 (47)	39 (55)	
Alive with recurrence	7 (8)	7 (13)	4 (6)	0.07
Died of disease	11 (12)	12 (23)	18 (25)	
Died of other causes	8 (9)	9 (17)	10 (14)	

[1]Differences between the three treatment groups using Fisher's exact test; mean age with analysis of variance; [2]Includes the T3 tumors with threatened circumferential margin involvement; [3]Circumferential margin, data available in 164 cases. RT: Radiotherapy.

JTT) blinded to clinical data. Squamous basal cells of anal epithelium included in some of the cases were used as a positive control for CD44v6. Using 5×, 10× and 20 × objectives, the immunostaining of CD44v6 in every slide was assessed using the following parameters: (1) the extent of expression (membranous and cytoplasmic staining scored separately); (2) the intensity of expression (membranous and cytoplasmic staining scored separately); and (3) the intratumoral pattern of membranous CD44v6 expression. The extent of expression within the whole tumor area was analyzed using four categories: 1 for immunopositivity < 5%, 2 for 5%-20%, 3 for 21%-50% and 4 for > 50%. The intensity of expression was analyzed using four categories: 1 for negative staining; 2 for weak staining (a faint immunopositivity in the membrane or cytoplasm); 3 for moderate staining (a clear immunopositivity in the membrane or cytoplasm); and 4 for strong staining (a pronounced immunostaining in the membrane or cytoplasm equivalent to that of the basal cells of anal squamous epithelium). The intratumoral pattern of membranous CD44v6 expression was classified into three categories: 1 for immunopositivity mainly in the tumor invasive front; 2 for immunopositivity mainly in the tumor central areas; and 3 for heterogenous immunopositivity (no apparent difference in CD44v6 expression between the invasive front and central areas of tumor).

For statistical purposes, categories 1 and 2 were studied as one group and categories 3 and 4 as another group when analyzing the extent and intensity of expression. With regard to the intratumoral pattern of membranous staining, categories 1 and 3 were studied as one group ("front-positive"), and category 2 as in its own group ("front-negative").

Evaluation of tumor growth pattern

In order to evaluate tumor growth pattern, hematoxylin-eosin and Ck-Pan stainings were scrutinized in each operative sample. Using the Jass' classification of the tumor growth pattern, tumours were appointed as "expanding" when the invasive margin was pushing or reasonably well circumscribed, and "infiltrating" when the tumor invaded in a diffuse manner with widespread penetration into adjacent normal tissues[23].

Evaluation of tumor regression grade

Tumor regression grade (TRG) after long-course RT was defined by a pathologist (Sundström JTT) as poor, moderate or excellent according to the modified Dworak and Rödel scales, as described recently[24]. Briefly, poor TRG was defined as minimal or no tumor regression after (chemo) RT. In case of poor response, many tumor cells remained after treatment. In tumors with moderate response, there were only some tumor cells or tumor cell groups left in the primary tumor, lymph nodes or perirectal fat. In tumors with excellent response, few or no tumor cells could be detected.

Statistical analysis

Statistical analysis were run using IBM SPSS® Statistics 19.0.1 for Windows software. Frequency tables were analyzed using the χ^2 test, with the likelihood ratio (LR) or Fisher's exact test for categorical variables. 2 × 2 tables were used to calculate odds ratio and 95% CI using the exact method. Fisher's exact test, Spearman's correlation and LR were used to assess the significance of the correlation between individual variables in univariate analysis. Inter-observer reproducibility of the assessments was tested using weighted κ. It was calculated using the intraclass correlation coefficient (ICC) test, in parallel mode with a two-way random model, using consistency assumption and the average-measures option to interpret the ICC (95% CI). The ICC of assessments was very good with weighted κ ranging from 0.70 to 0.90.

Univariate survival analysis for disease-free survival (DFS) and disease-specific survival (DSS) was based on the Kaplan-Meier method where stratum-specific outcomes were compared using log-rank (Mantel-Cox) statistics. To adjust for the covariates, a Cox proportional

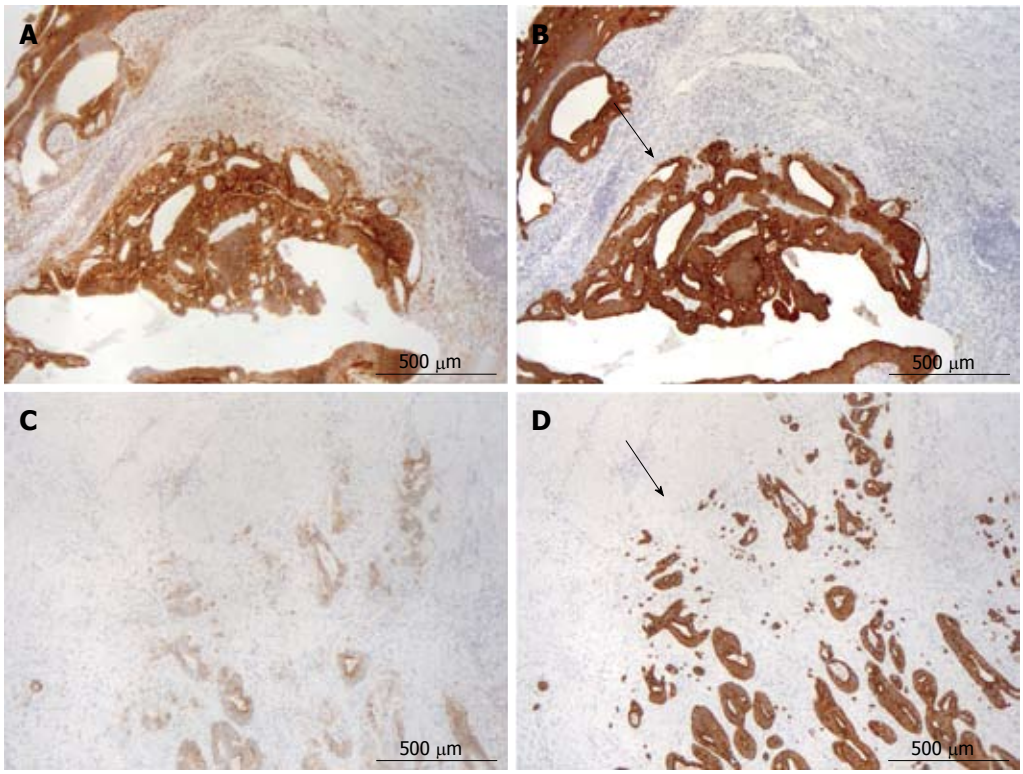


Figure 1 The immunohistochemistry for CD44 variant 6 and Pan-cytokeratin. A: The same tumor with "front-positive" membranous staining of CD44 variant 6 (CD44v6); B: Expanding growth pattern as shown with Pan-cytokeratin (Ck-Pan) staining; C: The same tumor with "front-negative" membranous staining of CD44v6; D: Infiltrating growth pattern as shown with Ck-Pan staining. Invasive front is indicated with arrows.

hazards regression model was used, covariates (as listed separately) being inserted using the enter mode. Of the variables significant in univariate analysis, two (circumferential margin, vascular invasion) were not included in the multivariate model because the data were incomplete. All statistical tests were two-sided and declared significant at a P value of < 0.05.

RESULTS

CD44v6 expression in tumors

Altogether, 177 out of 210 tumors (84%) showed membranous and 170 (81%) cytoplasmic CD44v6 expression. Both the extent and intensity of membranous and cytoplasmic staining were closely correlated ($r = 0.4$; $r = 0.31$, $P < 0.001$ for both). Of the 177 tumors with membranous CD44v6 positivity, 105 (59%) were "front-positive" (Figure 1A), usually showing an expanding growth pattern (Figure 1B), and 72 (41%) were "front-negative" (Figure 1C), usually showing an infiltrating growth pattern (Figure 1D).

Tumor growth pattern

In 206 tumors, a proper invasive front could be detected to evaluate tumor growth pattern. Altogether, 99 (48%) showed an expanding (Figure 1B) and 107 (52%) an infiltrating growth pattern (Figure 1D).

CD44v6 related to treatment groups and TRG

Only the extent of cytoplasmic staining was related to treatment group. Patients in the long-course RT group had less cytoplasmic CD44v6 expression in their tumors as compared to patients in the control group ($P = 0.002$). Within the long-course RT group, no differences in CD44v6 expression were seen according to concomitant chemotherapy. TRG after long-course RT, as analyzed also from the four tumors without IHC for CD44v6, was poor in 27 cases (51%), moderate in 14 cases (26%) and excellent in 12 cases (23%). No significant differences were seen in CD44v6 expression, as related to TRG.

CD44v6 related to clinicopathological variables

No significant differences were seen in the extent or intensity of CD44v6 expression concerning the variables listed in Table 1. With regard to the intratumoral pattern of membranous staining, "front-negative" was associated with narrower circumferential margin ($P = 0.01$), infiltrative tumor growth pattern ($P < 0.001$), and a greater risk for disease recurrence ($P = 0.01$ overall) as demonstrated in Table 2. No difference was seen in the number of patients receiving postoperative adjuvant chemotherapy between "front-positive" and "front-negative" groups ($P = 0.65$). Of the "front-positive" tumors, 37% were diagnosed before and 63% after 2005; the corresponding proportions for "front-negative" tumors being 22% and 78%, respectively ($P = 0.035$). No other significant correlations were seen between the staining pattern and clinicopathological variables, including PFS.

Table 2 Intratumoral staining pattern of membranous CD44 variant 6 expression related to treatment group and some clinicopathological variables *n* (%)

Variable (*n* = 177)	"Front-positive" (*n* = 105)	"Front-negative" (*n* = 72)	*P*[1]
Treatment group			
Short-course RT	46 (44)	32 (44)	
Long-course RT	24 (23)	14 (19)	0.87
Control	35 (33)	26 (36)	
Postoperative T			
T1-2	43 (41)	21 (29)	
T3	55 (52)	42 (58)	0.19
T4	7 (7)	9 (13)	
Postoperative N			
N0	68 (65)	40 (56)	
N1-2	36 (34)	31 (43)	0.37
Nx	1 (1)	1 (1)	
[2]Crm			
⩽ 2 mm	14 (18)	22 (38)	0.01
> 2 mm	64 (82)	36 (62)	
Tumor growth pattern			
Expanding	63 (60)	23 (32)	< 0.001
Infiltrating	42 (40)	49 (68)	
Disease-specific outcome			
Alive without recurrence	68 (65)	39 (54)	
Alive with recurrence	3 (3)	12 (17)	0.01
Died of disease	21 (20)	15 (21)	
Died of other causes	13 (12)	6 (8)	

[1]Pearson χ^2; *P* value for difference between "front-positive" and "front-negative" groups; [2]Circumferential margin, data available in 136 out of 177 cases. RT: Radiotherapy.

Table 3 Results of multivariate analysis

Variable	DFS			DSS		
	Adjusted HR	95% CI	*P*	Adjusted HR	95% CI	*P*
Sex						
Female (ref)	1.0			1.0		
Male	1.9	1.0-3.5	0.04	1.5	0.7-3.0	0.3
Age						
< 70 yr (ref)	1.0			1.0		
⩾ 70 yr	1.4	0.8-2.4	0.3	4.7	2.1-10.5	< 0.001
Postoperative T						
T1-2 (ref)	1.0			1.0		
T3	3.2	1.4-7.2	0.005	2.0	0.8-5.1	0.1
T4	5.9	2.0-17.0	0.001	4.8	1.4-17.0	0.02
Postoperative N						
N0 (ref)	1.0			1.0		
N1-2, Nx	2.0	1.1-3.7	0.02	0.8	0.4-1.7	0.6
[1]Postoperative grade						
G1 (ref)				1.0		
G2				1.3	0.4-3.9	0.7
G3				5.2	1.6-17.4	0.007
Growth pattern						
Expanding (ref)	1.0			1.0		
Infiltrating	1.0	0.5-1.8	0.9	0.8	0.4-1.7	0.6
[1]CD44v6 staining pattern						
"Front-positive" (ref)	1.0					
"Front-negative"	1.4	0.8-2.6	0.2			
Recurrence						
No (ref)				1.0		
Yes				74.3	17.0-326.7	< 0.001

[1]Not significant in univariate analysis and thus not included in multivariate model. Ref: Reference category; HR: Hazard ratio; DFS: Disease-free survival; DSS: Disease-specific survival; CD44v6: CD44 variant 6.

Recurrence and survival analysis

In univariate survival (Kaplan-Meier) analysis, no differences in DFS or DSS were seen according to the extent or intensity of CD44v6 expression, neither membranous nor cytoplasmic (data not shown). DFS ($P = 0.022$), but not DSS ($P = 0.68$), in patients with "front-negative" tumors was significantly shorter than in patients with "front-positive" tumors (Figure 2A and B). The same trend/difference was also seen in the short-course RT and control groups ($P = 0.058$ and $P = 0.024$ for difference in DFS, respectively) when analyzing treatment subgroups separately. With regard to tumor growth pattern, infiltrating tumors had shorter DFS ($P = 0.015$), and a tendency towards a shorter DSS ($P = 0.14$), as compared to expanding tumors.

The results of multivariate analysis are summarized in Table 3. The independent adverse prognostic factors for DFS were male sex, high postoperative T and the presence of positive lymph nodes, and those for DSS were patient age, postoperative T4, poor differentiation grade and disease recurrence.

DISCUSSION

Loss and gain of adhesive functions play an essential role in the progression of epithelial neoplasms[25]. In the present study, the expression of CD44v6, a cell surface mediator of cell-to-ECM adhesion[4], was systematically studied in a cohort of 214 primary rectal carcinomas treated with or without preoperative RT. The actual extent of CD44v6 expression was not associated with disease progression or outcome, but the analysis of its intratumoral staining pattern showed that patients with "front-negative" staining pattern had a significantly shorter DFS.

In our series, both membranous and cytoplasmic staining of CD44v6 was present in most cases, with a strong mutual correlation as reported earlier[8]. We found that preoperative RT affected cytoplasmic but not membranous CD44v6 expression. Although membranous CD44v6 is known to bind extracellular hyaluronate and growth factors[5], the biological significance of cytoplasmic CD44v6 is rather unknown[8]. In some studies it has been considered to have no functional roles[8], whereas in others, it has been related to neoplastic transformation[12,26] suggesting a role in loss of differentiation[26]. As preoperative RT has been shown to cause remarkable histological alterations[27], the reason for a decrease in cytoplasmic CD44v6 expression after RT could reflect these histological changes. However, further studies are required to elucidate this suggestion.

The prognostic value of CD44v6 expression, regarding the extent of expression, has been studied with inconclusive results in CRC[8,11,14]. In accordance with Morrin *et al*[14], we found no differences in disease progression indicators and patient outcome according to the

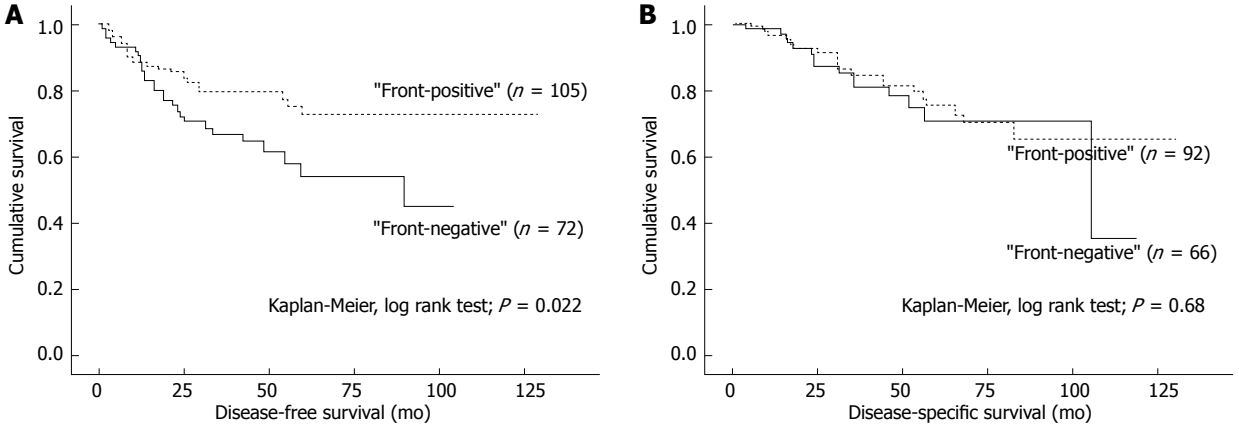


Figure 2 CD44 variant 6 related to disease-free and disease-specific survival in univariate analysis. Disease-free survival (A) and disease-specific survival (B) according to "front-positive" and "front-negative" membranous staining pattern of CD44 variant 6.

extent of CD44v6 expression. This finding is in contrast with some recent studies on rectal tumors, in which high membranous CD44v6 expression was shown to be associated with local recurrence[19] as well as shorter DFS[17-19] and DSS[17,18]. In contrast to these studies, we also included preoperatively treated tumors in our study to assess the possible relation of CD44v6 expression to TRG. This dissimilar study design may explain some of the discrepancy even though we found no differences in membranous CD44v6 expression parameters between the treatment or TRG-groups. In addition, the discrepancy may originate from differences in antibodies, cut-off points as well as disease subtypes in different studies as earlier discussed by Naor *et al*[28].

In addition to the extent of CD44v6 expression, we also assessed the intratumoral staining pattern of membranous expression. Given that tumor-host interaction at the invasive CRC front is thought to represent a dynamic interface between pro- and antitumor factors[20], it is not surprising that abnormalities in cell adhesion functions are seen in this compartment[29,30]. Although loss of membranous CD44v6 expression towards the CRC invasive front has been reported[8,12], our study is, to our knowledge, the first to assess the relation of membranous CD44v6 staining pattern to rectal cancer outcome. Furthermore, some of the previous studies are based on tissue microarray analyses, questioning the reliability of invasive front detection. Interestingly, "front-negative" tumors were related to more narrow postoperative circumferential margin and infiltrative tumor growth pattern as compared to "front-positive tumors" with apparent CD44v6 expression in the invasive front. Previously, Ishida[31] and Zlobec *et al*[8] with their co-workers have shown weak/negative CD44v6 expression to be related to infiltrating growth pattern, as assessed by its overall extent irrespective of its localisation. Our results may indicate that more important than the actual quantity of CD44v6 expression is, indeed, its localisation within the tumor. It is possible that "front-negative" tumors are more difficult for surgeon to resect with wide margins as they grow in a more diffuse manner, explaining the more narrow margins seen in these lesions.

Our observations lend support to the view of Zlobec *et al*[8], and Coppola *et al*[12], suggesting that the loss of membranous CD44v6 at the advancing edge of the tumor results in defective binding of the tumor cells to ECM, increasing their mobility and metastatic potential[8,12]. Further supporting this view, treatment with hyaluronic acid has recently been shown to delay the growth of residual colon carcinoma cells after chemotherapy[32]. Indeed, CD44v6 is known to have a higher affinity for hyaluronate, as compared to standard CD44[33], and, CD44v6-expressing tumors cells could be more effectively entrapped within the hyaluronic acid at the primary site[12]. In addition to binding with extracellular hyaluronate, membranous CD44v6 expression in the invasive front may also be important due to its intercellular adhesion properties, because loss of CD44v6 expression has been correlated to the loss of E-cadherin expression[8].

Importance of the localization of CD44v6 expression was further supported by the fact that "front-negative" tumors more often developed recurrent disease with shorter DFS as compared to patients with "front-positive" tumors. This was also observed in the subgroup analysis for the control group, and with a similar trend in the short-course RT group. No such appearance was seen in the long-course RT group, possibly due to the smaller number of cases available for this assessment. In some of the long-course RT cases, few tumor glands were left after treatment, and for this reason, no evident invasive front could be detected. However, despite this evident difference in DFS between the patients with "front-positive" and "front-negative" tumors, these two groups had practically identical DSS, which, due to the differences in the year of diagnoses between these two groups, could reflect the improvements in the treatment of recurrent rectal cancer.

The staining pattern of CD44v6 lost its significance in multivariate analysis. As in a number of other studies[34], we also found infiltrating tumor growth pattern to suggest an adverse DFS and a tendency towards a shorter DSS. The close correlation of CD44v6 staining

pattern with this prognostic factor may at least partly explain the lack of CD44v6 as an independent prognostic factor in our study. In addition, the other functions of this protein, such as binding of growth factors[35], may subvert the advantage of "front-positive" tumors.

Taken together, we found that tumors with "front-negative" CD44v6 membranous staining pattern were strongly related to invasive behavior. Our results substantiate the hypothesis that the lack, rather than overexpression, of membranous CD44v6 in the invasive tumor front contributes to rectal cancer progression. Although associated with a shorter DFS in univariate analysis, "front-negative" phenotype did not prove to be an independent prognostic factor in multivariate analysis, possibly confounded by other prognostic factors, including tumor growth pattern. We propose that analyzing the intratumoral staining pattern of membranous CD44v6 could offer a simple tool to predict the patients at increased risk for disease recurrence and thus in need of more aggressive postoperative treatment approaches and close monitoring.

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COMMENTS

Background

Colorectal cancer is a common malignancy. Radiotherapy alone or in combination with chemotherapy has been used to lower the risk of local recurrence. Still, a significant proportion of patients comes down with disease recurrence that is challenging to treat and causes harmful symptoms. New prognostic markers are constantly being studied to identify better the patients who need more aggressive treatment approaches in addition to surgery. CD44 variant 6 (CD44v6), a cell membrane receptor binding extracellular components, has been demonstrated to associate with rectal cancer progression and prognosis, but with discrepant results.

Research frontiers

In many studies, CD44v6 has been suggested to play a role in tumor progression and metastasis. The important area in the work was to study whether there is a difference in rectal cancer prognosis according to the intratumoral staining pattern of CD44v6. In contrast to previous studies, authors also included patients treated with radiotherapy before surgery.

Innovations and breakthroughs

The previous studies on the percentage and intensity of CD44v6 expression in rectal cancer have not been conclusive with regard to prognosis, because the overexpression of this protein has been associated with both favorable and adverse survival. Although alteration of CD44v6 expression in tumor invasive front has been demonstrated, its association with rectal cancer prognosis has not been studied in detail. Interestingly, the authors found that the patients with tumors showing CD44v6 expression in the invasive front presented with a longer disease-free survival time than did the patients whose tumors expressed protein more centrally. Moreover, the latter "front-negative" type was associated with infiltrating tumor growth and narrow tumor-free margin, both known to be adverse prognostic factors. Instead, the authors did not find the percentage and intensity of CD44v6 staining to be related to disease prognosis. The authors concluded that for rectal cancer progression and prognosis, more important than the actual amount of CD44v6 might be its distribution within the tumor.

Applications

The study suggests that patients with "front-negative" rectal cancer may need more aggressive treatment and monitoring after surgery.

Terminology

Prognostic factor is a situation or a condition, or a characteristic of a patient, that can be used to estimate the chance to recover from a disease or the risk for the disease recurring. Invasive front is the interface of tumor and host tissue; the deepest rim of cancerous tissue grown in adjacent non-cancerous tissues.

Peer review

In this work, the authors investigated the prognostic value of CD44v6 in patients treated with/without preoperative radiotherapy. The results are interesting and suggest that the lack of membranous CD44v6 in the rectal cancer invasive front could be used as a method to identify patients at increased risk for recurrent disease. The article is generally well written.

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BRIEF ARTICLE

Characteristics of intestinal pseudo-obstruction in patients with mitochondrial diseases

Yusuke Sekino, Masahiko Inamori, Eiji Yamada, Hidenori Ohkubo, Eiji Sakai, Takuma Higurashi, Hiroshi Iida, Kunihiro Hosono, Hiroki Endo, Takashi Nonaka, Hirokazu Takahashi, Tomoko Koide, Yasunobu Abe, Eiji Gotoh, Shigeru Koyano, Yoshiyuki Kuroiwa, Shin Maeda, Atsushi Nakajima

Yusuke Sekino, Masahiko Inamori, Eiji Yamada, Hidenori Ohkubo, Eiji Sakai, Takuma Higurashi, Hiroshi Iida, Kunihiro Hosono, Hiroki Endo, Takashi Nonaka, Hirokazu Takahashi, Tomoko Koide, Yasunobu Abe, Shin Maeda, Atsushi Nakajima, Gastroenterology Division, Yokohama City University School of Medicine, Yokohama 236-0004, Japan
Eiji Gotoh, Department of Medical Education, Yokohama City University School of Medicine, Yokohama 236-0004, Japan
Shigeru Koyano, Yoshiyuki Kuroiwa, Department of Neurology, Yokohama City University School of Medicine, Yokohama 236-0004, Japan
Author contributions: Sekino Y, Inamori M, Iida H, Nonaka T and Nakajima A performed the majority of clinical and treatment data collection; Takahashi H, Koide T, Abe Y, Gotoh E, Kuroiwa Y and Maeda S were involved in the editing of the manuscript; Yamada E, Ohkubo H, Sakai E, Higurashi T, Hosono K and Endo H analyzed the clinical data and participated in the design and coordination of the study; Sekino Y, Inamori M, Iida H, Nonaka T, Koyano S and Nakajima A designed the study and wrote the manuscript.
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Correspondence to: Dr. Masahiko Inamori, Gastroenterology Division, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. inamorim@med.yokohama-cu.ac.jp
Telephone: +81-45-7872640 Fax: +81-45-7843546
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Abstract

AIM: To reveal the frequency, characteristics and prognosis of chronic intestinal pseudo-obstruction (CIP) in mitochondrial disease patients.

METHODS: Between January 2000 and December 2010, 31 patients (13 males and 18 females) were diagnosed with mitochondrial diseases at our hospital. We conducted a retrospective review of the patients' sex, subclass of mitochondrial disease, age at onset of mitochondrial disease, frequency of CIP and the age at its onset, and the duration of survival. The age at onset or at the first diagnosis of the disorder that led to the clinical suspicion of mitochondrial disease was also examined.

RESULTS: Twenty patients were sub-classified with mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), 8 with chronic progressive external ophthalmoplegia (CPEO), and 3 with myoclonus epilepsy associated with ragged-red fibers (MERRF). Nine patients were diagnosed with CIP, 8 of the 20 (40.0%) patients with MELAS, 0 of the 8 (0.0%) patients with CPEO, and 1 of the 3 (33.3%) patients with MERRF. The median age (range) at the diagnosis and the median age at onset of mitochondrial disease were 40 (17-69) and 25 (12-63) years in patients with CIP, and 49 (17-81) and 40 (11-71) years in patients without CIP. During the survey period, 5 patients (4 patients with MELAS and 1 with CPEO) died. The cause of death was cardiomyopathy in 2 patients with MELAS, cerebral infarction in 1 patient with MELAS, epilepsy and aspiration pneumonia in 1 patient with MELAS, and multiple metastases from gastric cancer and aspiration pneumonia in 1 patient with CPEO.

CONCLUSION: Patients with CIP tend to have disorders that are suspected to be related to mitochondrial diseases at younger ages than are patients without CIP.

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Key words: Chronic intestinal pseudo-obstruction; Criteria; Mitochondrial disease; Mitochondrial encephalopathy; Lactic acidosis; Stroke-like episodes; Chronic progressive external ophthalmoplegia

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INTRODUCTION

Intestinal pseudo-obstruction was first reported by Dudley *et al*[1] in 1958, and refers to an uncommon disabling motility syndrome characterized by severe symptoms and signs of intestinal obstruction (abdominal pain, abdominal distention, nausea, and vomiting) and radiographic evidence of a dilated bowel in the absence of any mechanical obstruction. Pseudo-obstruction is primarily considered a small-intestine motility disorder, but it may occur in any portion of the gastrointestinal tract[2-6]. Furthermore, pseudo-obstruction may occur as an acute disease (Ogilvie syndrome[7]) or as a chronic remitting or persistent disorder, and chronic intestinal pseudo-obstruction (CIP) can be caused by and complicate many disorders[2]. Primary CIP can be sub-classified into visceral myopathy, visceral neuropathy and idiopathic CIP based on its histopathological manifestations. According to its etiology, secondary CIP can be categorized as disease-induced (such as connective tissue disorders, muscular dystrophies, infiltrative diseases, mitochondrial diseases, generalized nerve disease, endocrine disease, metabolic disease and others)[8-12] or drug-induced (antidepressant and anti-anxiety drugs, phenothiazines and others)[13-16].

Although an algorithm for the diagnosis of CIP was proposed by Rudolph *et al*[2] in 1997, diagnostic criteria for CIP have not yet been established in Japan or worldwide. Recently, Iida *et al*[17] proposed the diagnostic criteria for CIP shown in Table 1 and reported a diagnostic sensitivity of 85.9%.

Mitochondrial diseases are a heterogeneous group of disorders associated with mutations or deletions of nuclear or mitochondrial DNA[18-22]. Genetic mutations or deletions result in multisystem involvement associated with defects in the oxidative phosphorylation system and impaired production of ATP. The degree of organ dysfunction is contingent on the energy requirement of the organ and the proportion of mutated mitochondrial DNA in the organ[18]. Encephalomyopathy and cardiomyopathy are frequently encountered manifestations of mitochondrial diseases, and recently, gastrointestinal dysmotility has received attention[23-26]. Chinnery *et al*[27] reported that over 15% of patients with mitochondrial diseases complain of dysphagia or constipation, and a small percentage of the cases with constipation develop intestinal pseudo-obstruction. Although Amiot *et al*[28] reported that 19% of 80 patients with CIP had mitochondrial defects, the relationship between CIP and mitochondrial disease has not yet been conclusively established.

The aim of this study was to determine the frequency, characteristics, and prognosis of CIP in patients with mitochondrial diseases.

MATERIALS AND METHODS

Between January 2000 and December 2010, 33 patients were diagnosed with mitochondrial diseases at the Yokohama City University School of Medicine. Their clinical and treatment data were collected from their medical records and 2 patients were excluded due to insufficient clinical data.

Diagnosis of mitochondrial disease

The plasma and cerebrospinal fluid levels of lactate and pyruvate were measured at rest and then re-evaluated under exercise stress to ensure that the levels were within the normal ranges. Muscle biopsies and genetic analyses were performed in all patients. Examinations for other component disorders of mitochondrial dysfunction, such as glucose intolerance, electrocardiography, echocardiography, and brain magnetic resonance imaging were performed as needed.

Ethical approval

This study was conducted in accordance with the declaration of Helsinki, and with the approval of the Ethics Committee of Yokohama City University School of Medicine. We obtained written informed consents from each of the patients.

Statistical analysis

We conducted a retrospective review of each patient's sex, subclass of mitochondrial disease, age at the onset of mitochondrial disease, age at the establishment of the diagnosis of mitochondrial disease, frequency of CIP and the age at onset, and the duration of survival. The age at onset or at the first diagnosis of the disorder that led to the clinical suspicion of mitochondrial disease was also examined. As controls, we collected the data of 57 patients who were diagnosed with progressive muscular dystrophy at the Yokohama City University School of Medicine between January 2007 and December 2011. Five of the patients were excluded due to insufficient clinical data.

RESULTS

Thirty-one patients with mitochondrial diseases underwent detailed assessment (Table 2). The subjects comprised 13 males and 18 females and were sub-classified as having mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS; $n = 20$), chronic progressive external ophthalmoplegia (CPEO; $n = 8$), or myoclonus epilepsy associated with ragged-red fibers

Table 1 Criteria for the diagnosis of chronic intestinal pseudo-obstruction

Must include
One or more symptoms of ileus[1] onset at least 6 mo prior to diagnosis
One or both of following for the last 12 wk: (1) Abdominal pain; (2) Abdominal bloating
Dilatation and/or air-fluid levels of the intestine on abdominal X-ray, echo and/or computed tomography imaging
No evidence of structural disease (including findings of upper endoscopy, lower endoscopy, computed tomography, barium enema, and small-bowel follow-through) that could explain dilatation and/or air-fluid levels of the intestine
Supportive criteria
Congenital and/or onset under 15 years old must be excluded. Only adult onset is included
Surgical history within the 6 mo prior to diagnosis must be excluded to rule out Ogilvie syndrome, except surgery for CIP
To define CIP at two levels: Primary CIP or secondary CIP. Primary CIP consists of three types: the muscular type, neurogenic type and idiopathic type; Secondary CIP consists of two types: the systemic sclerosis (SSc) type and unclassified type
Family accumulation may exist
Neuropathy such as problems with urination may exist
Some psychosocial disorder may be present

[1]Symptoms of ileus include: Abdominal pain, nausea, vomiting, abdominal bloating, abdominal fullness, lack of defecation and/or passing gas. CIP: Chronic intestinal pseudo-obstruction.

Table 2 Age at the oncet of mitochondrial disease and chronic intestinal pseudo-obstruction

Sex	Subclass	Age at the onset of mitochondrial disease, yr	Age at the diagnosis of mitochondrial disease, yr	Age at the diagnosis of CIP, yr
F	MELAS	Difficulty in walking at 40	40	40
M	MELAS	Glucose intolerance at 13	57	46
M	MERRF	Difficulty in walking at 41	50	50
F	CPEO	Palpebral ptosis at 71	81	-
F	MELAS	Impaired hearing at 45	54	-
M	MELAS	Impaired hearing at 24	29	-
M	CPEO	Palpebral ptosis at 40	69	-
F	CPEO	Impaired eye movement at 30	52	-
F	MELAS	Impaired hearing at 25	40	43
M	CPEO	Palpebral ptosis at 49	49	-
F	MERRF	Muscular weakness at 46	54	-
M	MELAS	Impaired hearing at 47	52	-
M	MELAS	Epilepsy at 11	18	-
M	CPEO	Glucose intolerance at 40	67	-
M	MELAS	Impaired hearing at 24	31	-
F	MELAS	Impaired hearing at 12	17	25
F	MELAS	Impaired hearing at 54	60	-
F	MELAS	Epilepsy at 40	44	-
M	MELAS	Glucose intolerance and impaired hearing at 18	18	-
F	MELAS	-	23	-
F	MELAS	Chronic diarrhea at 26	40	-[1]
F	MELAS	Epilepsy at 18	18	26
F	MELAS	Difficulty in walking and hearing at 63	69	69
F	MELAS	Glucose intolerance at 33	42	55
M	CPEO	Palpebral ptosis at 16	44	-
M	MELAS	Epilepsy at 29	29	-
F	CPEO	Palpebral ptosis at 13	17	-
F	CPEO	Palpebral ptosis at 59	59	-
M	MELAS	Epilepsy at 21	21	34
F	MELAS	Glucose intolerance at 46	48	-
F	MERRF	Difficulty in walking at 20	50	-

[1]This patient died of uncontrolled epilepsy and aspiration pneumonia 5 mo after the onset of intestinal pseudo-obstruction and did not fulfill the diagnostic criteria. M: Male; F: Female; CIP: Chronic intestinal pseudo-obstruction; MERRF: Myoclonus epilepsy associated with ragged-red fibers; MELAS: Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes; CPEO: Chronic progressive external ophthalmoplegia.

(MERRF; n = 3). Of the 31 patients, 9 (28.1%) were diagnosed with CIP based on Nakajima's criteria, including 8 of the 20 (40.0%) patients with MELAS, 0 of the 8 (0.0%) patients with CPEO, and 1 of the 3 (33.3%) patients with MERRF. One patient died of uncontrolled epilepsy and aspiration pneumonia 5 mo after the onset of intestinal pseudo-obstruction and did not fulfill the diagnostic criteria.

The confirmable outcomes were as follows: in December 2010, 14 patients were receiving outpatient treatment, 12 patients had been transferred to chronic care facilities, and 5 patients (4 patients with MELAS and 1 with CPEO) had died. The cause of death was cardiomyopathy in 2 patients with MELAS, cerebral infarction in 1 patient with MELAS, epilepsy and aspiration pneumonia in 1 patient with MELAS, as previously described, and multiple metastases from gastric cancer and aspiration pneumonia in 1 patient with CPEO.

In radiographic examinations for the 9 patients with CIP, small intestinal distention was observed in 6 patients, and large intestinal distention was observed in 1 patients. Both small and large intestinal distention was observed in 2 patients.

The median age (range) at the establishment of the diagnosis of mitochondrial disease was 40 (17-69) years in patients with CIP and 49 (17-81) years in patients without CIP. The median age (range) at the onset or at the first diagnosis of the disorder that led to the suspicion of mitochondrial disease was 25 (12-63) years in patients with CIP and 40 (11-71) years in patients without CIP.

The symptoms of CIP were treated with laxative agents, antidiarrheal drugs, antiflatulence agents, mosapride, dimethicone, pantothenic acid, daikenchuto (Chinese herbal medicine), neostigmine or distigmine, and none of the patients required surgery (Table 3).

As controls, we collected the data of 57 patients with progressive muscular dystrophy (Table 4). The patients consisted of 2 patients with Duchenne muscular dystrophy, 7 patients with Becker muscular dystrophy, 4

Table 3 Characteristics of chronic intestinal pseudo-obstruction in patients with mitochondrial diseases

Sex	Subclass	Type of abdominal discomfort	Area of dilated intestine	Treatment
F	MELAS	Nausea, vomiting, diarrhea, constipation	Small intestine	PEG, neostigmine
M	MELAS	Diarrhea	Small intestine	Antidiarrheal drug
M	MERRF	Pain, diarrhea, distension	Small and large intestines	Mosapride, dimethicone, antiflatulent, daikenchuto, pantothenic acid
F	MELAS	Diarrhea, constipation, distension	Small and large intestines	Mosapride, daikenchuto, magnesium oxide
F	MELAS	Nausea, vomiting, diarrhea, constipation, pain	Small intestine	PEG, antiflatulent, magnesium oxide, antidiarrheal drug
F	MELAS	Nausea, vomiting, constipation, pain	Small intestine	Prostarmon, magnesium oxide, dimethicone, daikenchuto, mosapride, neostigmine, sodium picosulfate hydrate
F	MELAS	Nausea, vomiting, distension	Small intestine	Magnesium oxide, daikenchuto
F	MELAS	Nausea, vomiting	Large intestine	Daikenchuto, sennoside
M	MELAS	Nausea, vomiting, diarrhea, distension	Small intestine	Daikenchuto, mosapride, magnesium oxide, lansoprazole, neostigmine, dimechicone

M: Male; F: Female; CIP: Chronic intestinal pseudo-obstruction; MERRF: Myoclonus epilepsy associated with ragged-red fibers; MELAS: Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes; PEG: Polyethylene glycol.

Table 4 Characteristics of chronic intestinal pseudo-obstruction in patients with progressive muscular dystrophy

Subclass	No. of patients	No. of females	Age at the onset[1]	Age at the diagnosis[1]
Duchenne muscular dystrophy	2	0	1	2 (1-3)
Becker muscular dystrophy	7	0	10 (1-30)	19 (6-43)
Limb-girdle muscular dystrophy	4	1	22.5 (5-40)	49 (10-53)
Facio-scapulo-humeral muscular dystrophy	1	1	NS	NS
Myotonic dystrophy	38	18	30 (5-62)	39 (12-67)
Total	52	20	25 (1-62)	34.5 (1-67)

[1]Data is presented as median age (range). NS: Not stated. None of the patients with progressive muscular dystrophy in this study fulfilled the diagnostic criteria of chronic intestinal pseudo-obstruction.

patients with limb-girdle muscular dystrophy, 1 patient with facio-scapulo-humeral muscular dystrophy and 43 patients with myotonic dystrophy (5 patients were excluded due to insufficient clinical data). The median age (range) at the establishment of the diagnosis of progressive muscular dystrophy was 34.5 (1-67) years and the median age (range) at the onset of progressive muscular dystrophy was 25 (1-62) years. Only 2 of the patients died, while the others were alive at the last hospital visit. Although progressive muscular dystrophy has been considered as one of the etiologies of CIP, none of the patients with progressive muscular dystrophy in this study fulfilled the diagnostic criteria of CIP.

DISCUSSION

Although mitochondrial diseases have been considered as a potential cause of secondary CIP, the frequency of CIP in patients with mitochondrial diseases has not yet been well established. Gastrointestinal dysmotility (including CIP) may be caused by the accumulation of intracellular long-chain fatty acids, activation of extramitochondrial fatty acid oxidation pathways and excessive generation of reactive oxygen species leading to visceral myopathy, possibly as a result of impaired mitochondrial beta-oxidation[29]. Betts *et al*[30] reported that profound COX deficiency was found in the smooth muscle layers of all regions of the gastrointestinal tract in patients with the m.3243A>G mutation despite scarce evidence of morphologic abnormalities within the gastrointestinal tissues of these patients. Parsons *et al*[31] also reported that 80% of patients with MELAS and more than 60% of m.3243A>G carriers have 1 or more autonomic symptoms; gastrointestinal symptoms were especially common in the MELAS group, occurring in 66% of these patients and in almost 40% of the mutation carriers. In mitochondrial diseases, mitochondrial neurogastrointestinal encephalopathy (MNGIE), which is an uncommon autosomal recessive syndrome caused by the reduced activity of thymidine phosphorylase due to a mutation of the nuclear DNA, has also been reported to be particulary associated with CIP along with other symptoms such as malnutrition, progressive external ophthalmoplegia, ptosis, peripheral neuropathy, and leukoencephalopathy[32-34]. Although we have never encountered patients with MNGIE, this study revealed that patients with mitochondrial diseases develop complicating CIP at a relatively high frequency (28.1%), especially patients with MELAS (40.0%). We also examined the clinical data of patients with progressive muscular dystrophy, which is also a known cause of CIP, as controls and found that CIP occurs less frequently in patients with progressive muscular dystrophy than in patients with mitochondrial diseases (0% *vs* 28.1%).

Patients with CIP tend to have disorders, such as glucose intolerance, epilepsy, hearing impairment and palpebral ptosis,that are suspected to be related to mitochondrial diseases at younger ages than are patients without CIP. None of the patients in our study presented with CIP as the initial symptom of mitochondrial disease. This trend indicates that some patients with mitochondrial dysfunction will develop gastrointestinal dysmotility with disease progression, eventually leading

to the development of CIP.

The frequency of CIP among patients with relatively mild mitochondrial dysfunction who do not need advanced treatment at a hospital may also be smaller than that noted in this study. Most patients with mitochondrial diseases die of cardiomyopathy and encephalopathy before the development of severe CIP, and this finding is consistent with a previous report indicating that the degree of organ dysfunction depended on the energy requirement of the organ. Although CIP was not a major cause of death in patients with mitochondrial diseases, it often resulted in impaired oral intake or tube feeding and eventually the critical loss of the activities of daily life. Early detection and treatment of gastrointestinal dysmotility, including CIP, and the maintenance of the nutritional status may be efficacious in the management of patients with mitochondrial diseases.

There is still no sufficiently effective treatment for CIP today. A number of medicines were used in this study, and none were especially effective when comparing these treated patients with CIP patients without mitochondrial diseases. Surgery has been reported to be effective in patients with normal small intestinal motility[35], although surgery was not performed in this study (8 of 9 patients with CIP in this study had small intestine dysmotility).

Selection bias is one of the major limitations of our study. Patients receiving outpatient treatment at a university hospital like ours may have more severe symptoms than general patients presenting to primary care practitioners may have. This limitation could have resulted in an over-estimation of the CIP prevalence in patients with mitochondrial diseases. In addition, the 31 patients with mitochondrial diseases in this study included 20 patients with MELAS (64.5%), 8 patients with CPEO (25.8%) and 3 patients with MERRF (9.7%), indicating a larger prevalence of MELAS patients in our study compared with that from an epidemiological study conducted in Japan (64.5% *vs* 25.5%)[36]. This selection bias may also have contributed to an over-estimation of the CIP prevalence. Moreover, patients with MELAS often use antiepileptic drugs regularly, which may cause drug-induced intestinal pseudo-obstruction. In our study, 5 patients with CIP regularly used antiepileptic drugs (phenytoin, carbamazepine, carbamazepine and sodium valproate, and for 2 patients, carbamazepine and clonazepam). Although it may be difficult to precisely distinguish between mitochondrial and drug-induced gastrointestinal symptoms, the regular use of these antiepileptic drugs alone rarely results in CIP, and mitochondrial dysfunction was considered the main cause of the severe intestinal dysmotility of these 5 patients.

In conclusion, patients with mitochondrial diseases (especially MELAS) sometimes develop CIP. In cases characterized by the adequate control of fetal mitochondrial dysfunctions such as cardiomyopathy and encephalopathy, CIP may present as a prominent problem. The possibility of the development of gastrointestinal dysmotility, including CIP, should be considered at all stages of the clinical course of all patients with mitochondrial diseases.

COMMENTS

Background

Chronic intestinal pseudo-obstruction (CIP) is characterized by severe symptoms and signs of intestinal obstruction without mechanical obstruction. Although mitochondrial diseases have been considered as a potential cause of secondary CIP, the frequency, characteristics, and prognosis of CIP in patients with mitochondrial diseases has not yet been well established.

Research frontiers

Mitochondrial diseases are a heterogeneous group of disorders associated with mutations in or deletions of nuclear or mitochondrial DNA. The degree of organ dysfunction depends on the energy requirements of the organ and on the proportion of mitochondrial DNA in the organ. In the treatment of mitochondrial patients with CIP, current research now focuses on how mitochondrial dysfunction develops into CIP and what the differences between the patients with CIP and without CIP are.

Innovations and breakthroughs

The relationship between CIP and mitochondrial diseases has not yet been firmly established. In the present study, authors conducted a retrospective review of each patient's sex, subclass of mitochondrial disease, age at the onset of mitochondrial disease, age at the establishment of the diagnosis of mitochondrial disease, frequency of CIP and the age at its onset, and the duration of survival. The age at the onset or at the first diagnosis of the disorder that led to the clinical suspicion of mitochondrial disease was also examined.

Applications

The study results suggest that patients who have certain disorders, such as glucose intolerance, epilepsy, hearing impairment and palpebral ptosis, that are suspected to be related to mitochondrial diseases at younger ages tend to develop gastrointestinal dysmotility with disease progression, eventually leading to the development of CIP.

Terminology

CIP is an uncommon disabling motility syndrome characterized by severe symptoms and signs of intestinal obstruction (abdominal pain, abdominal distention, nausea and vomiting) and radiographic evidence of dilated bowels in the absence of any mechanical obstruction; Mitochondrial diseases are a heterogeneous group of disorders associated with mutations in or deletions of nuclear or mitochondrial DNA.

Peer review

This is a good descriptive study in which the authors retrospectively reviewed the frequency, characteristics and prognosis of chronic intestinal pseudo-obstruction in mitochondrial patients. The study revealed that patients with CIP tend to have disorders that are suspected to be related to mitochondrial diseases at younger ages than patients without CIP.

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BRIEF ARTICLE

Effect of composite yogurt enriched with acacia fiber and *Bifidobacterium lactis*

Yang Won Min, Sang Un Park, Yeon Sil Jang, Young-Ho Kim, Poong-Lyul Rhee, Seo Hyun Ko, Nami Joo, Sun Im Kim, Cheol-Hyun Kim, Dong Kyung Chang

Yang Won Min, Sang Un Park, Yeon Sil Jang, Young-Ho Kim, Poong-Lyul Rhee, Dong Kyung Chang, Division of Gastroenterology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea
Seo Hyun Ko, Nami Joo, Sun Im Kim, Department of Food and Nutrition, Sookmyung Women's University, Seoul 140-742, South Korea
Cheol-Hyun Kim, Department of Animal Resource and Science, Dankook University, Cheonan 330-714, South Korea
Author contributions: Min YW analyzed the data and wrote the draft manuscript; Park SU and Jang YS analyzed the data; Kim YH and Rhee PL provided the data and undertook critical revision of the manuscript; Ko SH, Joo N, Kim SI and Kim CH undertook critical revision of the manuscript and were involved in study design; and Chang DK designed the study.
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Correspondence to: Dong Kyung Chang, Professor, MD, PhD, Division of Gastroenterology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea. dkchang@skku.edu
Telephone: +82-2-34103409 Fax: +82-2-34106983
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Abstract

AIM: To investigate whether composite yogurt with acacia dietary fiber and *Bifidobacterium lactis* (*B. lactis*) has additive effects in irritable bowel syndrome (IBS).

METHODS: A total of 130 patients were randomly allocated to consume, twice daily for 8 wk, either the composite yogurt or the control product. The composite yogurt contained acacia dietary fiber and high-dose *B. lactis* together with two classic yogurt starter cultures. Patients were evaluated using the visual analog scale *via* a structured questionnaire administered at baseline and after treatment.

RESULTS: Improvements in bowel habit satisfaction and overall IBS symptoms from baseline were significantly higher in the test group than in the control group (27.16 *vs* 15.51, $P = 0.010$, 64.2 ± 17.0 *vs* 50.4 ± 20.5, $P < 0.001$; respectively). In constipation-predominant IBS, improvement in overall IBS symptoms was significantly higher in the test group than in the control group (72.4 ± 18.4 *vs* 50.0 ± 21.8, $P < 0.001$). In patients with diarrhea-predominant IBS, improvement in bowel habit satisfaction from baseline was significantly higher in the test group than in the control group (32.90 *vs* 7.81, $P = 0.006$).

CONCLUSION: Our data suggest that composite yogurt enriched with acacia fiber and *B. lactis* has greater therapeutic effects in patients with IBS than standard yogurt.

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Key words: Acacia dietary fiber; *Bifidobacterium lactis*; Irritable bowel syndrome; Probiotics; Yogurt

Peer reviewers: Dr. Ian David Wallace, Shakespeare Specialist Group,181 Shakes-perare Rd., Milford, 1309 Auckland, New Zealand; Gabrio Bassotti, Professor, Clinical and Experimental Medicine, Gastroenterology and Hepatology Section, Ospedale Santa Maria della Misericordia, Piazza Menghini, 06156 Perugia, Italy

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INTRODUCTION

Irritable bowel syndrome (IBS) is a functional bowel disorder characterized by symptoms of abdominal pain or discomfort associated with disturbed defecation, and is one of the most common gastrointestinal problems[1-3]. The pathogenesis of IBS is incompletely understood but over the past few years, there has been an emergence of new etiological hypotheses. These include gastrointestinal infection, low-grade infiltration and activation of mast cells in the intestinal mucosa with consequent release of bioactive substances, modification of small bowel and colonic microflora, changes related to the brain-gut axis, and altered serotonin metabolism[4-8]. These new views of the pathogenesis of IBS have changed the approach to IBS treatment[9]. Among several treatment options, the use of probiotics seems to be promising[10].

Probiotics are live microorganisms with a vast array of therapeutic potential for gastrointestinal disease[11,12]. They have been studied and used in many gastrointestinal disorders, with growing evidence for use in pouchitis, *Clostridium difficile* colitis, antibiotic-associated diarrhea, inflammatory bowel disease, and IBS. The emerging multifactorial pathophysiological paradigm of IBS may create adjunctive probiotic therapeutic opportunities[10,13,14]. The most widely studied organisms are *Bifidobacterium lactis* (*B. lactis*) and *Lactobacillus spp.*[15]. *B. lactis* survives complete transit through the digestive tract and is recovered live in stools in large quantities relative to the quantity initially ingested[16,17]. Daily consumption of fermented milk containing *B. lactis* was reported to improve gastrointestinal transit and digestive comfort, alleviate bloating, and increase stool frequency[18,19].

Acacia gum is extensively used as a food additive. It is a complex polysaccharide, that is primarily indigestible, not degraded in the intestine, but fermented in the colon[20]. Acacia fiber is made from acacia gum. Recently, its prebiotic properties, meaning it selectively stimulates the intestinal flora, were described and a synergy for bifidogenicity was observed with the combination of other prebiotics (fructo-oligosaccharide) and acacia gum. In addition, because acacia fiber is slowly fermented, it may attenuate the side effects of fermentation. Intestinal gas production resulting from fermentation can induce abdominal symptoms[21]. Dietary fiber is also commonly used in the treatment of patients with IBS[22]. Although dietary fiber does not appear to be useful as a sole treatment of IBS, it may have a limited role in empiric therapy, especially if constipation is the most significant symptom[23,24].

The aim of this study was to investigate whether a composite yogurt enriched with acacia dietary fiber and *B. lactis* had additive therapeutic effects in patients with IBS when compared with standard yogurt.

MATERIALS AND METHODS

Study population

A total of 130 patients were recruited at Samsung Medical Center, Seoul, South Korea. They were male and female patients between 18 and 70 years of age who met the Rome Ⅲ criteria[25] for the diagnosis of IBS.

Exclusion criteria were as follow: (1) endocrine disorders, neurological disorders, cardiovascular disorders, inherited neuromuscular disorders, malignant tumors, renal failure (serum creatinine ≥ 3.0 mg/dL), and liver cirrhosis (child class B and C); (2) current use of medications that potentially influence bowel habits such as medications for constipation, antidiarrheal drugs, and medications that can cause constipation, including anticholinergic drugs (e.g., anticonvulsants, antihistamines, antipsychotic and neuroleptic agents, anti-Parkinson agents, and antidepressants), narcotic pain medications (e.g., codeine), resins (e.g., cholestyramine), and metal ions and inorganic compounds (e.g., aluminum-, calcium-, and iron-containing antacids); (3) age > 55 years without a history of a sigmoidoscopy or colonoscopy performed in the previous 5 years; (4) abnormal results on a sigmoidoscopy or colonoscopy and abdominal radiological tests performed in the previous 2 years; and (5) any previous abdominal surgery.

Study protocol

This was a randomized, double-blind, controlled trial. Simple randomization was performed using a random number table. Potentially eligible patients answered a structured questionnaire at baseline. At that time, they were provided with a standardized explanation of questions and symptom definitions. In addition, patients were evaluated *via* a full review of their clinical history including complete blood count, serum chemistry, and thyroid function test. Clinically significant abnormalities in any of the latter tests resulted in exclusion from the study. Thereafter, eligible patients were randomly allocated to consume two bottles daily (one at breakfast, one at dinner) for 8 wk of either the composite yogurt (test product) or the control product. At the end of the study, patients were again administered a questionnaire. The use of any medication that potentially influences bowel habits was prohibited for 7 d prior to consumption of the products.

The study protocol was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board at Samsung Medical Center, Seoul, South Korea (No. 2010-07-223). All subjects provided written informed consent before inclusion in the study.

Study products

The test product was a yogurt containing high-dose *Bifidobacterium animalis subsp. lactis Bb-12* (*B. animalis subsp. lactis Bb-12*) (≥ 10^{11} cfu/bottle), *Bifidobacterium* enhancer, and acacia dietary fiber, together with the two classic yogurt starter cultures, *Streptococcus thermophilus* (≥ 3×10^9 cfu/bottle) and *Lactobacillus acidophilus* (*L. acidophilus*) (≥ 10^9 cfu/bottle).

The control product was a traditional yogurt contain-

ing *B. animalis subsp. lactis Bb-12* ($\geq 10^{10}$ cfu/bottle), no extra-functional ingredients (*Bifidobacterium* enhancer and acacia dietary fiber), and two yogurt starter cultures, *Streptococcus thermophilus* ($\geq 3 \times 10^{9}$ cfu/bottle) and *L. acidophilus* ($\geq 1 \times 10^{9}$ cfu/bottle). Both the test and control products were without added flavor and had similar appearance, color, texture, and taste. Each bottle contained either 150 mL of test or control product and was provided by the Namyang Dairy Products Co. Ltd. (Seoul, South Korea).

Assessments

Each patient was evaluated using a structured questionnaire at baseline and after 8 wk treatment. At baseline, the questionnaire assessed age, sex, height, body weight, IBS subtype[26], abdominal symptoms (abdominal pain/discomfort, abdominal distension/bloating, and flatulence), and bowel habits (frequency and duration of defecation, urgency, straining, feeling of incomplete defecation, stool consistency, bowel habit satisfaction, and discomfort related to daily life). Abdominal pain/discomfort, abdominal distension/bloating, bowel habit satisfaction, and discomfort related to daily life were evaluated using the visual analog scale (VAS, 0 = no symptoms, 25 = mild, 50 = moderate, 75 = severe, and 100 = very severe). Abdominal pain/discomfort, flatulence, and defecation were assessed by frequency. The stool consistency was determined using the Bristol stool scale[27].

At the end of the treatment, abdominal symptoms and bowel habits were assessed by the self-administration of the questionnaire that had the same questions as the one at baseline. In addition, the improvement of overall IBS symptoms was evaluated using the VAS (0 = aggravated, 25 = no change, 50 = slightly improved, 75 = much improved, 100 = very much improved).

Statistical analysis

It was determined that 50 patients per group were required for a power of 85% and two-sided significance at 5% in detecting a between-group effect of 0.2 in the improvement of overall IBS symptoms. To ensure the inclusion of at least 50 patients per group, 65 per group were ultimately recruited in order to account for a potential withdrawal rate of 25%.

Baseline demographic data were compared between groups using Student's *t* test, Pearson's χ^2 test, or Fisher's exact test, as appropriate. Changes in the symptom scores after treatment for each group were assessed using a paired *t* test, McNemar's test, and a generalized estimating equation. A two-sided *P* value < 0.05 was considered statistically significant. Statistical analysis was performed using PASW Statistics 18 for Windows (SPSS, Inc, Chicago, IL, United States).

RESULTS

Baseline characteristics and response to treatment

A total of 130 patients were enrolled and randomized either to the test group ($n = 65$) or the control group ($n = 65$). Thirteen patients discontinued the study and were lost to follow-up, and 117 (58 in the test group and 59 in the control group) completed the study. Using the Rome Ⅲ criteria, 35.0% of patients were classified as constipation-predominant IBS (IBS-C), 29.9% as diarrhea-predominant IBS (IBS-D), 8.5% as mixed IBS (IBS-M), and 26.5% as unsubtyped IBS. Table 1 shows baseline characteristics and symptom scores of patients in the test and control groups. At baseline, the distributions of age, sex, body mass index, and IBS subtype were similar between the groups, and the baseline scores for abdominal symptoms and bowel habits did not differ.

Table 2 summarizes the changes in the study parameters after 8 wk treatment in both the test and control groups and differences between the groups. Bowel habit satisfaction improved more in the test group than in the control group (change from baseline of 27.16 *vs* 15.51, $P = 0.010$), and the improvement in overall IBS symptoms was significantly higher in the test group than in the control group (64.2 ± 17.0 *vs* 50.4 ± 20.5, $P < 0.001$). The scores for abdominal pain/discomfort, abdominal distension/bloating, and discomfort related to daily life also improved more in the test group than in the control group, but the improvements did not significantly differ between the groups. The improvements in straining and feeling of incomplete evacuation did not differ between the groups. Defecation duration and frequency, flatulence, and urgency did not improve after treatment. The change in stool consistency was different in the two groups. Stool consistency did not change in the test group but became softer in the control group. There were no significant adverse events reported throughout the study.

Analysis by IBS subtype

A subgroup analysis was performed to determine the effects of the test product on each IBS subtype. In the IBS-C group (Table 3), the improvement in overall IBS symptoms was significantly higher in the test group than in the control group (72.4 ± 18.4 *vs* 50.0 ± 21.8, $P < 0.001$), and the difference between the two groups was greater than that between the test and control groups including all of the study patients (64.2 ± 17.0 *vs* 50.4 ± 20.5, $P < 0.001$). However, bowel habit satisfaction did not differ between the two groups. Defecation frequency and feeling of incomplete evacuation, which did not improve in the study patients overall, improved in the test and control groups (change from baseline of 1.79, $P = 0.002$ and 1.96, $P = 0.032$; change from baseline -42.1%, $P = 0.021$ and -31.8%, $P = 0.016$, respectively) although the improvements did not significantly differ between the groups. Stool consistency became softer in both groups without a significant difference between the test and control groups (change from baseline of 0.789 *vs* 1.09, $P = 0.386$). In the IBS-D group (Table 4), bowel habit satisfaction improved more in the test group than in the control group (change from baseline of 32.90 *vs*

Table 1 Baseline characteristics and symptom scores (mean ± SD)

	Test group (n = 58)	Control group (n = 59)	P value
Age (yr)	37.43 ± 10.27	34.24 ± 8.67	0.720
Male:female n (%)	17 (29.3):41 (70.7)	18 (30.5):41 (69.5)	0.887
BMI (kg/m^2)	21.96 ± 3.01	21.28 ± 2.50	0.184
Subtype of IBS n (%)			0.919
Constipation	19 (32.8)	22 (37.3)	
Diarrhea	19 (32.8)	16 (27.1)	
Mixed	5 (8.6)	5 (8.5)	
Unsubtyped	15 (25.9)	16 (27.1)	
Abdominal symptoms			
Abdominal pain or discomfort (VAS)	34.05 ± 18.55	33.05 ± 19.94	0.779
Frequency of abdominal pain or discomfort/d	1.73 ± 2.03	1.21 ± 1.17	0.089
Abdominal distension or bloating (VAS)	44.40 ± 21.99	39.83 ± 20.82	0.281
Flatulence/d	5.19 ± 4.01	4.98 ± 2.88	0.749
Bowel habit			
Defecation frequency/wk	6.38 ± 5.98	5.69 ± 3.87	0.458
Defecation duration (min)	8.68 ± 6.35	8.85 ± 5.59	0.881
Urgency n (%)	23 (39.7)	22 (37.3)	0.792
Straining n (%)	39 (67.2)	37 (62.7)	0.608
Feeling of incomplete defecation n (%)	39 (67.2)	44 (74.6)	0.382
Stool consistency (BSS)	3.95 ± 1.64	3.54 ± 1.59	0.159
Bowel habit satisfaction (VAS)	32.33 ± 17.53	31.95 ± 18.22	0.909
Discomfort related to daily life (VAS)	36.21 ± 22.54	30.93 ± 17.58	0.160

BMI: Body mass index; IBS: Irritable bowel syndrome; VAS: Visual analog scale; BSS: Bristol stool scale.

Table 2 Study parameters at week 8 and changes from baseline in all patients (mean ± SD)

	Test group (n = 58)				Control group (n = 59)				P value[1]
	Baseline	wk 8	Δ wk 8	P value	Baseline	wk 8	Δ wk 8	P value	
Abdominal symptoms									
Abdominal pain or discomfort (VAS)	34.05 ± 18.55	12.93 ± 14.99	-21.12	0	33.05 ± 19.94	16.5 ± 17.7	-16.53	< 0.001	0.26
Frequency of abdominal pain or discomfort/d	1.73 ± 2.03	0.84 ± 0.83	-0.89	0.004	1.21 ± 1.17	0.7 ± 0.8	-0.48	0.001	0.214
Abdominal distension or bloating (VAS)	44.40 ± 21.99	25.86 ± 18.1	-18.53	0	39.83 ± 20.82	28.8 ± 21.2	-11.02	< 0.001	0.096
Flatulence/d	5.19 ± 4.01	5.69 ± 5.08	0.5	0.391	4.98 ± 2.88	5.5 ± 4.0	0.51	0.266	0.991
Bowel habits									
Defecation frequency/wk	6.38 ± 5.98	7.23 ± 4.28	0.85	0.289	5.69 ± 3.87	6.7 ± 4.2	1.04	0.052	0.843
Defecation duration (min)	8.68 ± 6.35	7.47 ± 4.95	-1.22	0.07	8.85 ± 5.59	6.7 ± 3.9	-2.12	< 0.001	0.301
Urgency	23 (39.7%)	16 (27.6%)	-7	0.118	22 (37.3%)	22 (37.3%)	0	1	0.146
Straining	39 (67.2%)	22 (37.9%)	-17	0	37 (62.7%)	20 (33.9%)	-17	0.002	0.959
Feeling of incomplete evacuation	39 (67.2%)	19 (32.8%)	-20	0	44 (74.6%)	23 (39.0%)	-21	< 0.001	0.815
Stool consistency (BSS)	3.95 ± 1.64	3.72 ± 1.02	-0.22	0.274	3.53 ± 1.59	3.88 ± 1.15	0.36	0.047	0.118
Bowel habit satisfaction (VAS)	32.33 ± 17.53	59.48 ± 19.21	27.16	0	31.95 ± 18.22	47.5 ± 20.1	15.51	< 0.001	0.01
Discomfort related to daily life (VAS)	36.21 ± 22.54	21.98 ± 19.91	-14.22	0	30.93 ± 17.58	22.9 ± 17.5	-8.05	0.007	0.199
Improvement in overall IBS symptoms (VAS)		64.2 ± 17.0				50.4 ± 20.5			< 0.001

[1]Comparison between both groups. IBS: Irritable bowel syndrome; VAS: Visual analog scale; BSS: Bristol stool scale.

7.81, P = 0.006), and the difference between the two groups was greater than the difference between patients in the overall analysis (change from baseline of 27.16 *vs* 15.51, P = 0.010). However, the improvement in overall IBS symptoms did not differ between the two groups. Abdominal pain/discomfort scores improved more in the test group than in the control group, and the improvement was nearly significant (change from baseline of -23.68 *vs* -9.38, P = 0.050). Stool consistency became harder in both groups without a significant difference between the test and control groups (change from baseline of -1.26 *vs* -0.63, P = 0.738). In the IBS-M group, the improvements in abdominal symptoms, bowel habits, and overall IBS symptoms did not significantly differ between the test and control groups.

DISCUSSION

Several clinical studies have demonstrated the efficacy of probiotics for the treatment of patients with IBS. Some studies have also been performed in populations of certain IBS subtypes. A study by Kim *et al*[28] evaluated the effect of VSL#3, a combination of probiotics that contains live bacteria including *Bifidobacterium*, *Lactobacillus*, and *Streptococcus salivarius ssp. thermophilus,* on gastrointestinal transit and symptoms in IBS-D. With VSL#3 treatment, the decrease in bloating was of borderline significance, but there was no effect on gastrointestinal transit

Table 3 Study parameters at week 8 and changes from baseline in constipation-predominant irritable bowel syndrome patients (mean ± SD)

	Test group (n = 19)				Control group (n = 22)				P value[1]
	Baseline	wk 8	Δ wk 8	P value	Baseline	wk 8	Δ wk 8	P value	
Abdominal symptoms									
Abdominal pain or discomfort (VAS)	28.95 ± 20.90	9.21 ± 12.39	-19.74	0.001	37.50 ± 25.30	15.91 ± 18.17	-21.59	0.001	0.8
Frequency of abdominal pain of discomfort/d	1.50 ± 1.17	0.89 ± 0.88	-0.61	0.032	1.27 ± 1.56	0.68 ± 0.78	-0.6	0.029	0.979
Abdominal distension or bloating (VAS)	44.74 ± 21.38	25.00 ± 16.67	-19.74	0.007	38.64 ± 22.79	26.14 ± 16.33	-12.5	0.031	0.393
Flatulence/wk	6.05 ± 5.03	6.13 ± 4.35	0.08	0.952	5.52 ± 3.34	6.02 ± 4.95	0.5	0.577	0.785
Bowel habit									
Defecation frequency/wk	3.82 ± 2.08	5.61 ± 3.54	1.79	0.002	3.43 ± 1.55	5.39 ± 3.97	1.96	0.032	0.872
Defecation duration (min)	12.26 ± 7.33	9.66 ± 5.28	-2.61	0.106	10.84 ± 6.49	6.59 ± 4.07	-4.25	< 0.001	0.358
Urgency	4 (21.1%)	4 (21.1%)	0	1	3 (13.6%)	6 (27.3%)	3	0.375	0.336
Straining	18 (94.7%)	11 (57.9%)	-7	0.016	18 (81.8%)	12 (54.5%)	-6	0.146	0.321
Feeling of incomplete evacuation	15 (78.9%)	7 (36.8%)	-8	0.021	16 (72.7%)	9 (40.9%)	-7	0.016	0.776
Stool consistency (BSS)	2.26 ± 0.45	3.05 ± 0.85	0.789	0	2.18 ± 0.66	3.27 ± 1.08	1.09	0.001	0.386
Bowel habit satisfaction	30.26 ± 19.68	56.58 ± 20.14	26.32	0	27.27 ± 18.76	44.32 ± 21.73	17.05	0.004	0.21
Discomfort related to daily life (VAS)	34.21 ± 22.38	18.42 ± 16.33	-15.79	0.014	36.36 ± 21.45	27.73 ± 15.25	-13.64	0.015	0.782
Improvement in overall IBS symptoms (VAS)		72.4 ± 18.4				50.0 ± 21.8			< 0.001

[1]Comparison between both groups. IBS: Irritable bowel syndrome; VAS: Visual analog scale; BSS: Bristol stool scale.

Table 4 Study parameters at week 8 and changes from baseline in diarrhea-predominant irritable bowel syndrome patients (mean ± SD)

	Test group (n = 19)				Control group (n = 16)				P value[1]
	Baseline	wk 8	Δ wk 8	P value	Baseline	wk 8	Δ wk 8	P value	
Abdominal symptoms									
Abdominal pain or discomfort (VAS)	32.89 ± 14.56	9.21 ± 14.93	-23.68	0	29.69 ± 10.08	20.3 ± 18.8	-9.38	0.083	0.05
Frequency of abdominal pain or discomfort/d	2.45 ± 3.18	0.63 ± 0.83	-1.82	0.036	1.25 ± 0.71	0.91 ± 0.94	-0.34	0.245	0.117
Abdominal distension or bloating (VAS)	43.42 ± 23.34	25.00 ± 20.41	-18.42	0.012	35.94 ± 15.73	29.69 ± 20.85	-6.25	0.164	0.146
Flatulence/wk	4.63 ± 2.36	4.08 ± 2.67	-0.55	0.503	4.66 ± 2.15	5.50 ± 3.38	0.84	0.255	0.212
Bowel habit									
Defecation frequency/wk	10.55 ± 8.18	8.79 ± 4.46	-1.76	0.381	9.09 ± 4.19	9.09 ± 4.12	0	1	0.451
Defecation duration (min)	6.66 ± 5.57	6.58 ± 4.98	-0.08	0.938	8.00 ± 4.75	7.03 ± 3.81	-0.97	0.3	0.52
Urgency	10 (52.6%)	8 (42.1%)	-2	0.625	12 (75.0%)	11 (68.8%)	-1	1	0.867
Straining	8 (42.1%)	3 (15.8%)	-5	0.063	7 (43.8%)	2 (12.5%)	-5	0.063	0.707
Feeling of incomplete evacuation	13 (68.4%)	6 (31.6%)	-7	0.039	12 (75.0%)	5 (31.3%)	-7	0.016	0.826
Stool consistency (BSS)	5.42 ± 1.17	4.16 ± 0.38	-1.26	0.001	5.50 ± 1.10	4.88 ± 1.09	-0.63	0.036	0.738
Bowel habit satisfaction	31.58 ± 18.34	64.47 ± 19.21	32.9	0	40.63 ± 15.48	48.44 ± 17.00	7.81	0.173	0.006
Discomfort related to daily life (VAS)	36.84 ± 24.11	23.68 ± 25.65	-13.16	0.163	29.69 ± 13.60	28.13 ± 17.97	-1.56	0.751	0.292
Improvement in overall IBS symptoms (VAS)		61.8 ± 17.4				51.6 ± 14.3			0.07

[1]Comparison between both groups. IBS: Irritable bowel syndrome; VAS: Visual analog scale; BSS: Bristol stool scale.

or other individual IBS symptoms. A study by Guyonnet *et al*[19] assessed the effects of fermented milk containing *B. animalis* DN-173 010 in IBS-C and reported improvements in the health-related quality-of-life discomfort score and bloating symptoms, as well as increased stool frequency. Another study demonstrated the effects of a fermented milk product containing *B. lactis* DN-173-010 on abdominal distension and gastrointestinal transit in IBS-C[29]. In the present study, the therapeutic effect of the composite yogurt differed according to IBS subtype; in IBS-C, overall IBS symptoms were improved, and in IBS-D, the improvement in bowel habit satisfaction was prominent. However, irrespective of IBS subtype, the new composite yogurt had additive therapeutic effects on bowel habit satisfaction and overall IBS symptoms among the entire IBS study sample when compared with standard yogurt.

Lactobacilli and *bifidobacteria*, alone or in combination, have been used for the treatment of IBS in many clinical studies. A study by Sinn *et al*[14] reported that 4 wk treatment with *L. acidophilus*-SDC 2012, 2013 was associated with a reduced score for abdominal pain or discomfort compared to baseline. O'Mahony *et al*[30] performed a study in patients with IBS and grouped them into three different treatment arms. The patients received *Lactobacillus salivarius* UCC4331, *Bifidobacterium infantis* (*B. infantis*) 35624, or placebo, but only *B. infantis* alleviated IBS symptoms. This was associated with a normalization of the anti-inflammatory to proinflammatory cytokine ratio (interleukin-10/interleukin-12), suggesting an immune-modulating role for *B. infantis*. A larger study by Whorwell *et al*[31] evaluating different doses of *B. infantis* 35624 was performed in 362 women with IBS. The participants were randomized to receive either the placebo or encapsulated *B. infantis* at a dose of 10^6, 10^8 or 10^{10} cfu for 4 wk. *B. infantis* at a dose of 10^8 cfu was significantly superior to placebo and all other doses in improving IBS symptoms. However, the 10^{10} dose was associated

with significant formulation problems. From only these two studies, it is not possible to determine which strain is most effective and the optimal dose. Nevertheless, to date, *Bifidobacterium* seems to be one of the most important probiotics, and a *B. infantis* dose of at least 10^8 cfu may be appropriate for the treatment of IBS. Given this fact, it can be deduced that at a *Bifidobacterium* dose > 10^8 cfu, the therapeutic effects would increase in proportion to the dose up to a certain point. In this study, we used *B. lactis*, which has been shown to have therapeutic effects in IBS in several studies[18,19,29,32]. Our results also showed that the new composite yogurt (containing > 10 times the *B. lactis* of the control product and *Bifidobacterium* enhancer) was associated with a significant improvement in bowel habit satisfaction and overall IBS symptoms, although acacia fiber was also added to the test product and it was difficult to attribute the results to one or the other component. To determine the optimal dosage, another trial evaluating the effects of yogurt containing *B. lactis* at different dosages is necessary.

Dietary fiber accelerates whole gut transit time and increases daily stool weight and the proportion of unformed stool, and its efficacy in alleviating constipation has been confirmed in patients with IBS[33]. Therefore, dietary fiber is frequently recommended for IBS[22]. A study by Choi *et al*[24] evaluated the additive effects of probiotic fermented milk containing dietary fiber in IBS-C patients, compared to plain probiotic fermented milk, and dietary fiber had additive benefits for the symptoms of constipation, especially in IBS-C. However, in a study by Francis *et al*[34], fiber was found to exacerbate all symptoms of IBS. Dietary fiber is classified into soluble and insoluble fiber, which have different effects on global IBS-related symptoms[35]. Soluble fiber delays gastric emptying and nutrient absorption from the small bowel; it is used to delay gastric emptying and improve glycemic control in diabetes, as well as to alleviate constipation. Insoluble fiber has little effect on gastric emptying and small bowel transit; it markedly accelerates colonic transit and is frequently used as a laxative[36]. For our study, we used acacia fiber, which is soluble, and that is thought to be the reason the composite yogurt containing acacia fiber was associated with improvement in IBS symptoms among IBS-C patients.

The present study had some limitations. First, the follow-up period was relatively short. It would have been useful to know how participants were faring after using the composite yogurt for 6 and 12 mo. Next, this study was limited by the lack of a placebo group because the control product was not a placebo. Finally, we did not investigate participants' dietary factors, which may be more prevalent in IBS-D. Nevertheless, this was a well-designed trial with an appropriate number of patients. Furthermore, additive effects of high-dose *B. lactis* and acacia dietary fiber were clearly noted with respect to bowel habit satisfaction and overall IBS symptoms. Among IBS subtypes, overall IBS symptoms were more improved in IBS-C; in IBS-D, bowel habit satisfaction was more improved. In conclusion, a new composite yogurt had greater therapeutic effects in patients with IBS than standard yogurt had. Further studies are needed to determine the most effective probiotic strain, dosage, and duration of therapy.

COMMENTS

Background

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal problems. The new views of the pathogenesis of IBS have changed the approach to IBS treatment. Among several treatment options, the use of probiotics seems to be promising. Dietary fiber is also commonly used in the treatment of patients with IBS.

Research frontiers

The authors investigated whether a composite yogurt enriched with acacia dietary fiber and *Bifidobacterium lactis* (*B. lactis*) had additive therapeutic effects in patients with IBS when compared with standard yogurt.

Innovations and breakthroughs

This study was a well-designed prospective clinical trial with an appropriate number of patients. Additive effects of acacia dietary fiber and high-dose *B. lactis* were clearly noted with respect to bowel habit satisfaction and overall IBS symptoms.

Applications

The study results suggest that composite yogurt enriched with acacia fiber and *B. lactis* has greater therapeutic effects in patients with IBS than standard yogurt has.

Terminology

Probiotics are live microorganisms with a vast array of therapeutic potential for gastrointestinal disease.

Peer review

This study is very informative for clinicians because IBS is one of the most common gastrointestinal problems and clinicians frequently encounter problems with treating IBS patients. In addition, the results have scientific relevance for understanding the pathogenesis of the disease.

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BRIEF ARTICLE

Reduction of gastrointestinal motility by unilateral thyroparathyroidectomy plus subdiaphragmatic vagotomy in rats

Jun Ho Lee, Oh Deog Kwon, Seon Ho Ahn, Keun Han Choi, Ji Hye Park, Seoul Lee, Bong Kyu Choi, Kyu Yong Jung

Jun Ho Lee, Keun Han Choi, Ji Hye Park, Seoul Lee, Bong Kyu Choi, Kyu Yong Jung, Department of Pharmacology, Wonkwang University School of Medicine, Jeonbuk 570-749, South Korea
Oh Deog Kwon, Department of Clinical Pathology, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, South Korea
Seon Ho Ahn, Department of Internal Medicine, Wonkwang University School of Medicine, Jeonbuk 570-749, South Korea
Author contributions: Lee JH conducted the surgery; Kwon OD performed the biochemistry and analyzed the data; Ahn SH designed the study; Choi KH analyzed the data; Park JH, Lee S and Choi BK compiled the data; Jung KY wrote the paper.
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Correspondence to: Kyu Yong Jung, DVM, PhD, Department of Pharmacology, Wonkwang University School of Medicine, 344-2 Shinyong-dong, Iksan, Jeonbuk 570-749, South Korea. wkuniv@wonkwang.ac.kr
Telephone: +82-63-8506796 Fax: +82-63-8506764
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Abstract

AIM: To investigate whether the combined methods of unilateral thyroparathyroidectomy (TPX) and subdiaphragmatic vagotomy (VAX) can be adapted for rats and used as a reliable method to produce a rat model of long-term reduction of gastrointestinal (GI) motor function.

METHODS: Male Sprague-Dawley rats were randomly divided into 3 groups, normal, sham-operated and unilateral TPX plus VAX. The TPX plus VAX rats received VAX 7 d after application of TPX, and dietary intake and fecal output were then measured daily for 1 wk. After completion of the experiments, gastric emptying and small bowel transit were measured *in vivo*, and the contractile responses of colonic strips to excitatory and inhibitory neurotransmitters were estimated using isometric force transducers *in vitro*.

RESULTS: In comparison with normal and sham-operated rats, rats which received unilateral TPX plus VAX showed a significant decrease in body weight and in fecal pellet number and weight throughout the entire week. Application of TPX plus VAX to rats markedly delayed gastric emptying and small bowel transit. In TPX plus VAX rats, the longitudinal muscles of the proximal colon showed a significant reduction in contractile responses to acetylcholine (5×10^{-6} mol/L), and a dramatic attenuation of contractile responses was also observed in both the longitudinal and circular muscles of the distal colon. However, the spontaneous contractility of the colonic strips from TPX plus VAX rats was not significantly affected by treatment with *N*-nitro-*L*-arginine-methyl ester (0.1 mol/L).

CONCLUSION: The results indicate that unilateral TPX plus VAX reduced the motor function of the GI tract in rats, and the reduced gut motility is likely mediated, at least in part, by inhibition of the excitatory neurotransmitter system.

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Key words: Unilateral thyroparathyroidectomy; Subdiaphragmatic vagotomy; Gastric emptying; Small bowel transit; Rat

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INTRODUCTION

Vagal nerves and thyroid hormones regulate the physiological functions of the gastrointestinal (GI) tract. The subdiaphragmatic vagal nerve and its branches innervate the GI tract from the lower esophageal sphincter to the upper GI tract in humans[1,2], and the extensive distribution of vagal nerves to the gut plays an important role in the regulation of many digestive functions including food intake, gastric accommodation and GI motility[1,3]. Therefore, abnormalities in the subdiaphragmatic vagal nerves pathologically mediates the development of several GI diseases including dyspepsia and constipation[1,3,4]. In addition, hyper- and hypo-thyroidism also gives rise to a variety of GI symptoms including abnormal gastric secretion and GI motility[5,6]. The effects of hypothyroidism on the GI tract seem to be multi-factorial with alterations in neuroregulation, and a reduction in peristaltic activity being the most frequent GI complaint in patients with hypothyroidism[7].

It has been suggested that the vagal nerves predominantly act on the stomach and small intestine rather than the lower GI tract, and that subdiaphragmatic vagotomy (VAX) results in a transient slowing of the rate of gastric emptying and transit of the upper small intestine[8-10]. In our preliminary studies, rats which received subdiaphragmatic VAX transiently showed delayed gastric emptying and small bowel transit after surgery. Additionally, it has been reported that a complete depletion of thyroid hormones by bilateral thyroparathyroidectomy (TPX) in rats can induce a severe tetany which has a high mortality rate[11]. We also observed a similar phenomenon in preliminary studies, where bilateral TPX caused an extremely high fatality rate $\geqslant$ 90%) of rats within 48 h after surgical application. Taken together, we consider that bilateral TPX or subdiaphragmatic VAX alone is not a reliable and reproducible method to induce a long-term reduction in upper GI motility.

Therefore, we hypothesized that in comparison with bilateral TPX or subdiaphragmatic VAX, a combined method (TPX plus VAX) of unilateral TPX and subdiaphragmatic VAX might be a better strategy to provide a long-term reduction in spontaneous motility of the entire GI tract, but with reduced postoperative mortality. To examine this hypothesis, this study measured the effects of unilateral TPX plus VAX on the alteration of GI motility using rats, which are not only a popular laboratory animal for biomedical research, but also are a good model for studying the pharmacological properties of medicines, and for investigating the pathophysiological characteristics of human GI dysfunction[12,13].

MATERIALS AND METHODS

Animals and experimental groups

Male Sprague-Dawley rats (6-wk old) were obtained from Samtako BioKorea (Kyungki, South Korea) and acclimatized for 1 wk with free access to a solid normal rodent diet (Samyang Co., Kyungki, South Korea) and drinking water under a temperature of 23 ℃ ± 2 ℃, a relative humidity of 50%-60% and a 12-h/12-h dark-light cycle. After acclimatization, rats were randomly divided into 3 groups: normal, sham-operated and unilateral TPX plus VAX groups. Each group comprised 7-9 rats. The experimental procedures employed in this study are illustrated in Figure 1. All experiments were conducted in accordance with the institutional guidelines established by the Wonkwang University Commolittee for the Care and Use of Laboratory Animals.

Unilateral thyroparathyroidectomy

Unilateral TPX was carried out according to the previous method with a slight modification[14]. In brief, rats (7-wk old) were anesthetized using chloropent (5 mg/kg *ip*) for deep anesthesia, and thyroparathyroid glands on the right side were exposed by a mid-line incision of the neck under aseptic conditions and excised under stereomicroscopic observation. For sham-operated rats, the operation was performed using the same procedures except with no dissection of the thyroparathyroid glands. After surgery, animals were individually housed for 1 wk in cages with bedding of wood shavings.

Subdiaphragmatic vagotomy

At 7 d after unilateral TPX, subdiaphragmatic VAX was carried out according to a previous report with a slight modification[15]. Briefly, rats (8-wk old) were anesthetized using chloropent (5 mg/kg *ip*), and the abdomen around the stomach was opened under aseptic conditions. The ventral and dorsal branches of the subdiaphragmatic vagal nerves were cut (0.5 cm above the gastroesophageal junction). In sham-operated rats, the vagal trunks were similarly exposed, but without cutting of the vagal nerves. At the end of surgery, the completeness of VAX was verified by gastric responses to electrical stimulation, and inspection of the vagal nerve endings using a stereomicroscope as described in previous reports[8,15]. After dissecting the vagal nerves, animals were individually acclimatized for 1 d in wood-bedded cages and then housed in stainless-steel metabolic cages throughout the entire experimental period. Body weight, dietary intake, and fecal pellet number and weight were measured daily (9:00 am) for 7 d.

Measurement of gastric emptying

Gastric emptying was measured according to the previous method with a slight modification[16]. At 8 d after subdiaphragmatic VAX, rats were starved overnight but with drinking water available *ad libitum*. Each animal was sacrificed by cervical dislocation 10 min after oral ad-

ministration of a test meal (1 mL) containing 0.05% (w/v) phenol red in aqueous carboxymethyl cellulose (4.5%, w/v), and the stomach was immediately removed, followed by clamping of the gastro-esophageal and gastro-duodenal junctions. The stomach was rinsed with phosphate-buffered saline (PBS, pH 7.4) and dissected in 20 mL 0.1 mol/L NaOH. The stomach, containing phenol red solution, was shaken for 30 min and centrifuged at 3000 *g* for 10 min. To precipitate proteins, 0.5 mL of supernatant was added to 20% trichloroacetic acid (50 mL) and mixed vigorously. The samples were centrifuged at 10 000 *g* for 30 min, and 0.3 mL supernatant was mixed with 0.5 mol/L NaOH (0.4 mL) to develop the color. The absorbance of samples at 558 nm wavelength was measure with a spectrophotometer (U-2000; Hitachi, Tokyo, Japan). Gastric emptying was estimated from the following formulation: (amount of residual phenol red/phenol red present in stomach immediately after administration) × 100.

Measurement of small bowel transit

To examine the spontaneous motility of the small intestine, a small bowel transit test was measured an established method[17]. Briefly, rats at 8 d after application of subdiaphragmatic VAX were starved overnight but with drinking water available *ad libitum*. Each animal was orally administered 1 mL of a charcoal meal, 10% (w/v) activated charcoal in aqueous Arabic gum (5%, w/v), and sacrificed by cervical dislocation 20 min later. The gastro-duodenal and ileo-cecal junctions were tied, and the entire small intestine was then quickly removed to avoid stretching. After rinsing the intestine with PBS, small bowel transit was estimated by comparing the distance travelled by the charcoal meal from the pyloric sphincter to the total length of the small intestine from the pyloric sphincter to the ileo-cecal junction. The distance travelled by the charcoal meal was expressed as a percentage of the total length of the small intestine.

Preparation of colonic strips

At 8 d after subdiaphragmatic VAX, rats were starved overnight but with drinking water available *ad libitum* and sacrificed by CO_2 asphyxiation and cervical dislocation. The entire proximal and distal colons were removed, and the luminal contents were flushed out using Krebs-Ringer bicarbonate buffer (composition 118 mmol NaCl, 4.7 mmol KCl, 1.2 mmol KH_2PO_4, 1.2 mmol $MgSO_4$, 2.6 mmol $CaCl_2$, 25 mmol $NaHCO_3$ and 11.5 mmol *D*-glucose, pH 7.4). Strips (1 cm length) of proximal colon at a distance of 1 cm from the ileo-cecal junction and distal colon and at a distance of 1 cm from the anus were dissected. The intact colonic strips were longitudinally or circularly mounted in an organ bath (10 mL) containing Krebs-Ringer bicarbonate buffer bubbled with 5% CO_2/95% O_2 and maintained at 37 ℃. One end of each colonic strip was tied with suture silk and fixed to the bottom of the organ bath, and the other end was connected to an isometric force transducer (Grass Technologies, West Warwick, RI).

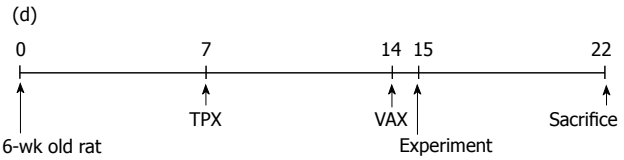


Figure 1 Schematic diagram of experiments to examine gastrointestinal motility after sequential application of unilateral thyroparathyroidectomy and subdiaphragmatic vagotomy. TPX: Thyroparatyroidectomy; VAX: Subdiaphragmatic vagotomy.

Measurement of colonic contractility

Colonic contractility was measured as described in our previous report[18]. Briefly, after the colonic strips were allowed to equilibrate for 30-60 min with washout every 10 min, 1 g of tension was slowly applied to the tissues before starting the experiments. The strips were treated with 0.5 mol of acetylcholine (ACh) or 0.1 mmol of *N*-nitro-*L*-arginine methyl ester (L-NAME) for 10 min, and the contractile responses of the colonic strips prepared from the unilateral TPX plus VAX rats to ACh and L-NAME were estimated and compared with the colonic contractility in strips of normal animals. Isometric contractile responses from the force transducer were measured by a biological recording system equipped with an amplifier (PowerLab 4/25, AD Instruments, Colorado Springs, CO, United States). The mean tensions and amplitudes of colonic contraction produced under resting and drug-treated conditions were measured over a period of 10 min.

Measurement of serum thyroxine contents

To estimate the change of serum thyroxine (T_4) level caused by unilateral TPX, total serum T_4 level at 15 d after application of unilateral TPX were measured using the T_4 enzyme-linked immunosorbent assay kit (GenWay Biotech; San Diego, CA, United States). Blood samples were collected from the abdominal vein at the time of sacrifice, and serum was obtained by centrifugation at 3000 *g* for 10 min. Serum samples were stored at -70 ℃ until T_4 analysis.

Statistical analysis

All results are presented as mean ± SE. One-way analysis of variance was performed to compare the statistical significance of multi-groups, followed by *post hoc* analysis by the Student-Newman-Keuls test for comparison of 2 groups. $P < 0.05$ was considered statistically significant.

RESULTS

General observation and serum thyroxine levels

No deaths occurred in the rats which received unilateral TPX plus VAX. In these rats, an obvious dilatation of the stomach and significant residual luminal content in the GI tract were found, but marked morphological changes

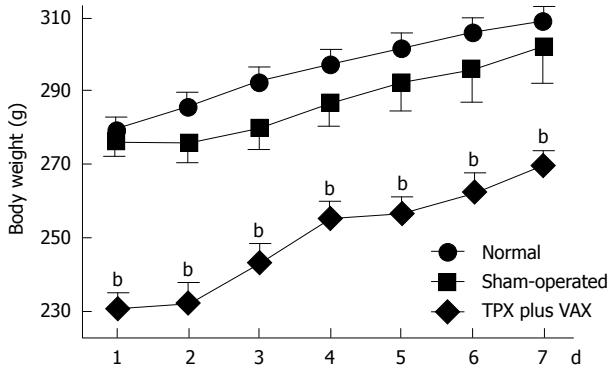


Figure 2 Effects of unilateral thyroparatyroidectomy plus subdiaphragmatic vagotomy on body weight gain in rats. Body weights were measured for 7 d from the day after subdiaphragmatic vagotomy (VAX). Each point represents the mean ± SE of 7-9 rats. $^{b}P < 0.01$ *vs* normal and sham-operated groups. TPX: Thyroparatyroidectomy.

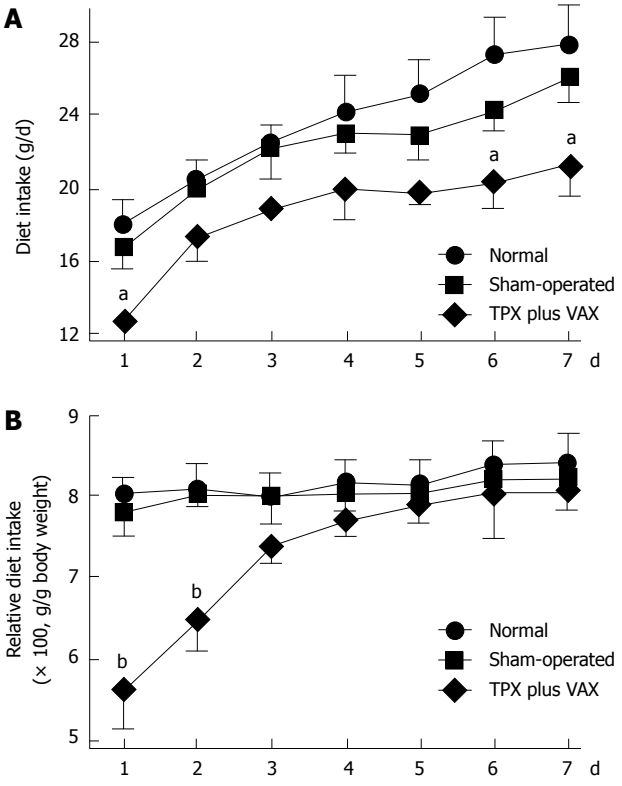


Figure 3 Changes in daily dietary intake in the thyroparatyroidectomy plus subdiaphragmatic vagotomy rats. Daily dietary intake (A: Absolute; B: Relative to body weight) was measured for 7 d from the day after subdiaphragmatic vagotomy (VAX). Each point represents the mean ± SE of 7-9 rats. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal and sham-operated groups. TPX: Thyroparatyroidectomy.

of the gut were not observed. There were no significant differences in visible features and behaviors among the normal (4.74 ± 0.85 g/dL), sham-operated (4.97 ± 1.14 g/dL) and unilateral TPX plus VAX rats (2.86 ± 0.98 g/dL). Serum T_4 levels in the unilateral TPX plus VAX rats were not significantly lower than those in the normal and sham-operated animals.

Body weight and dietary intake

As shown in Figure 2, body weights of unilateral TPX plus VAX rats were significantly lower than those of normal and sham-operated animals throughout the entire experimental period. However, there was no significant difference in body weights between normal and sham-operated rats. Daily dietary intake of the unilateral TPX plus VAX rats was not significantly lower than in normal and sham-operated animals throughout the entire experimental period (Figure 3A). The relative daily dietary intake in unilateral TPX plus VAX rats transiently decreased, but almost completely returned to normal levels after 4 d (Figure 3B).

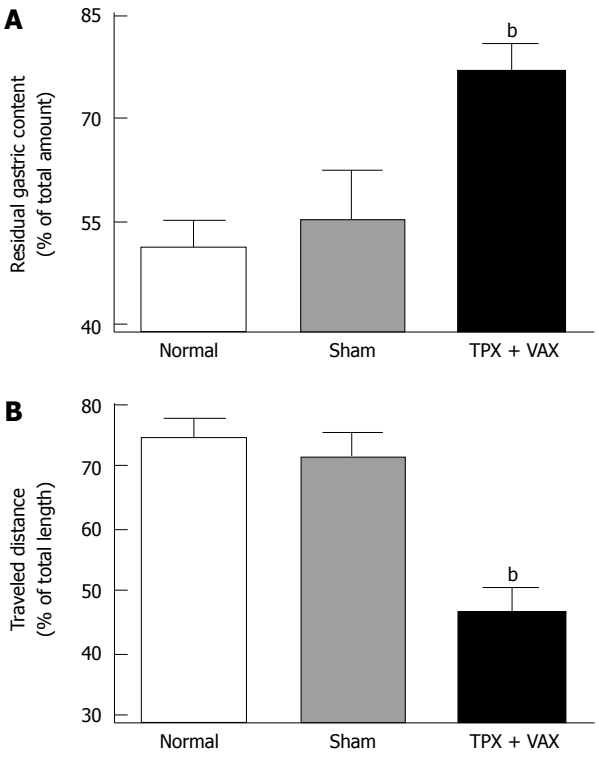


Figure 4 Effects of thyroparatyroidectomy plus subdiaphragmatic vagotomy on gastric emptying and small bowel transit in rats. Gastric emptying (A) and small bowel transit (B) was measured 8 d after subdiaphragmatic vagotomy (VAX). Each point represents the mean ± SE of 7-9 rats. $^{b}P < 0.01$ *vs* normal and sham-operated groups. TPX: Thyroparatyroidectomy.

Gastric emptying and small bowel transit

The motor functions of upper GI tracts were estimated by the gastric emptying test and small bowel transit *in vivo*, and the obtained results are shown in Figure 4. In comparison with normal and sham-operated rats, the residual gastric contents of unilateral TPX plus VAX rats were significantly elevated (Figure 4A), and the traveled distances of the luminal content in the small intestines of unilateral TPX plus VAX rats were markedly shortened (Figure 4B).

Defecatory function

Defecatory function was determined by measuring fecal pellet numbers and weights, and the obtained results are shown in Figure 5. In comparison with normal and sham-operated rats, daily fecal pellet numbers of unilateral TPX plus VAX rats were significantly reduced

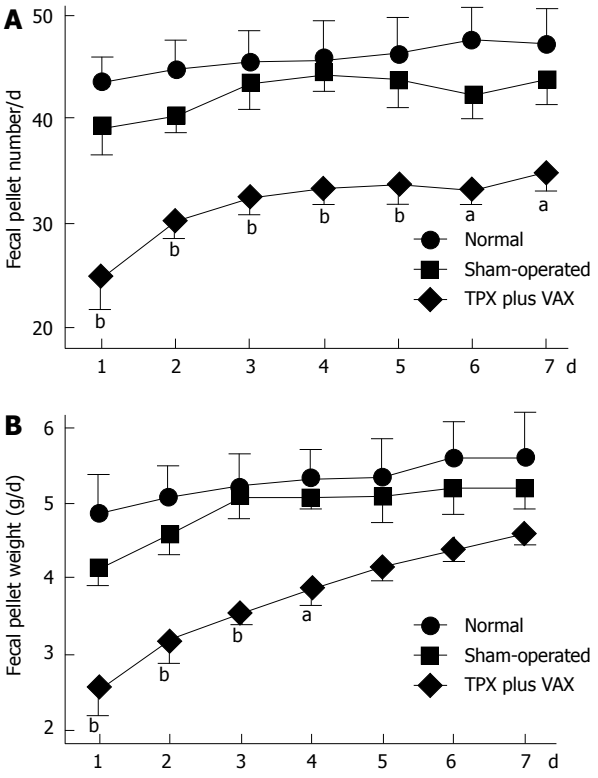


Figure 5 Effects of thyroparatyroidectomy plus subdiaphragmatic vagotomy on defecation of rats. Fecal pellet numbers (A) and weights (B) were measured for 7 d from the day after subdiaphragmatic vagotomy (VAX). Each point represents the mean ± SE of 7-9 rats. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal and sham-operated groups. TPX: Thyroparatyroidectomy.

throughout the entire experimental periods (Figure 5A), and their daily fecal weights were markedly reduced for the first 4 d of the experimental period (Figure 5B).

Colonic contractility

To examine changes in the spontaneous contractility of colonic smooth muscles, the contractile responses of intact colonic strips to ACh (0.5 mol) and L-NAME (0.5 mmol) were measured. The representative tracings obtained for proximal and distal colons are shown in the Figures 6A and 7A, respectively. In comparison with normal rats, the contractile responses to ACh (0.5 mol), both tension and amplitude, of the proximal colon longitudinal muscles prepared from the unilateral TPX plus VAX rats were significantly attenuated, whereas no significant changes were observed in the circular muscles (Figure 6B). L-NAME-induced contractility of the proximal colon did not significantly differ between the normal and unilateral TPX plus VAX rats (Figure 6C). Additionally, ACh-induced contractions of the distal colonic longitudinal and circular muscles prepared from the unilateral TPX plus VAX rats were significantly reduced compared with those of normal animals (Figure 7B), whereas the contractile responses of colonic strips to L-NAME did not significantly differ between normal and unilateral TPX plus VAX rats (Figure 7C).

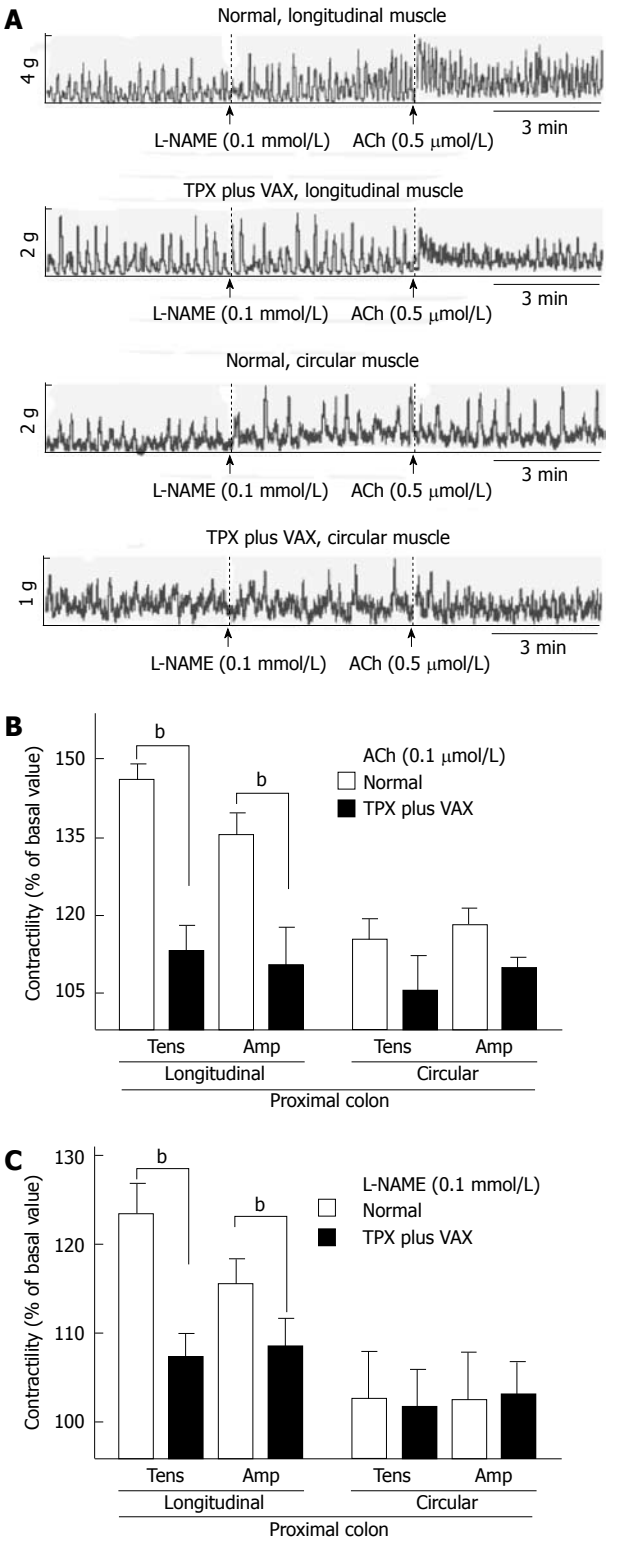


Figure 6 Reduced contractile activity of the proximal colonic muscles in the thyroparatyroidectomy plus subdiaphragmatic vagotomy rats. Animals were sacrificed at 8 d after subdiaphragmatic vagotomy (VAX), and intact proximal colonic strips prepared from the age-matched normal and thyroparatyroidectomy (TPX) plus VAX rats were longitudinally or circularly mounted in a 10 mL organ bath. The contractile responses of colonic strips to acetylcholine (Ach) and *N*-nitro-*L*-arginine methyl ester (L-NAME) were measured by isometric force transducers. Figures show the representative tracings (A) and contractile responses to ACh (B) and L-NAME (C). Each point represents the mean ± SE of 7-9 rats. $^{b}P < 0.01$ *vs* normal and sham-operated groups.

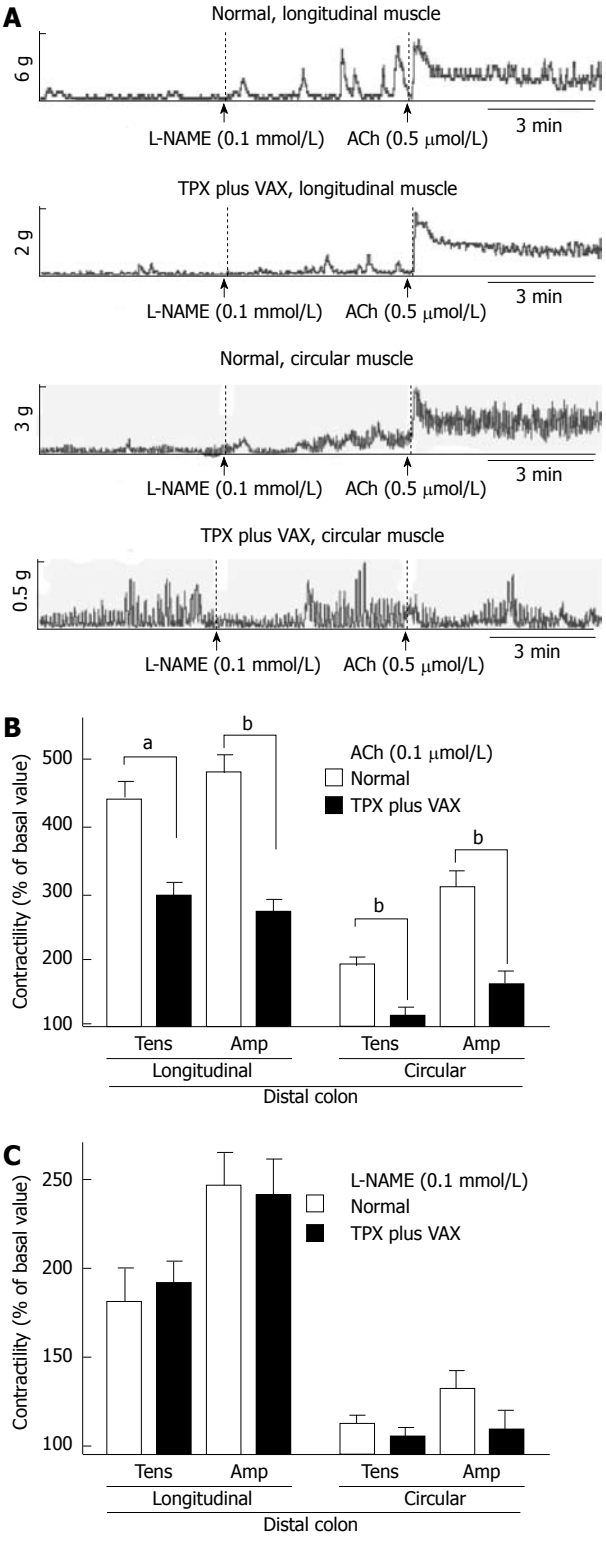


Figure 7 Reduced contractile activity of distal colonic muscles in the thyroparatyroidectomy plus subdiaphragmatic vagotomy rats. Animals were sacrificed at 8 d after subdiaphragmatic subdiaphragmatic vagotomy (VAX), and intact distal colonic strips prepared from the age-matched normal and thyroparatyroidectomy (TPX) plus VAX rats were longitudinally or circularly mounted in a 10 mL organ bath. The contractile responses of colonic strips to acetylcholine (Ach) and N-nitro-L-arginine methyl ester (L-NAME) were measured by isometric force transducers. Figures show the representative tracings (A) and contractile responses to ACh (B) and L-NAME (C). Each point represents the mean ± SE of 7-9 rats. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal and sham-operated groups.

DISCUSSION

To produce a rat model of a long-term reduction of the physiological motility of the entire GI tract, a partial depletion of thyroparathyroid hormones and denervation of subdiaphragmatic vagal nerves were sequentially applied to rats. In the rats receiving unilateral TPX plus VAX, an obvious dilatation of the stomach and significant residual luminal contents of GI tracts were found without marked morphological changes of the gut, and gastric emptying and small bowel transit were significantly delayed. The rats also showed a significant retardation of defecatory function. Additionally, we observed a significant reduction in distal colonic contractile responses to ACh, but not to L-NAME. These findings possibly indicate that a partial depletion of thyroparathyroid hormones and denervation of subdiaphragmatic vagal nerves decrease the spontaneous motility of the entire GI tracts through a reduction in the physiological activity of excitatory neurotransmitter systems. These dysfunctions are likely the pathological characteristics of the GI diseases related to reduced GI motility in humans.

Although a variety of animal models has been developed to examine the pathological mechanisms involved in the reduction of GI motility and to evaluate the pharmacological properties of drugs[19-22], it is unfortunately difficult to translate the findings of these animal models to human GI diseases. Although pathological characteristics of the bilateral TPX- or subdiaphragmatic VAX-induced animal models have been intensively investigated[1,3,6], the precise values for the combined method of unilateral TPX and subdiaphragmatic VAX are entirely, to our knowledge, lacking. Moreover, in the preliminary studies, we observed several scientific and methodological limitations of bilateral TPX or subdiaphragmatic VAX alone. A complete depletion of thyroparathyroid hormones by bilateral TPX caused an extremely high fatality rate (≥ 90%) within 48 h after surgery, and subdiaphragmatic VAX transiently (less than 3 d after surgery) reduced only gastric emptying and small bowel transit, but not colonic transit. These results led us to consider that bilateral TPX or subdiaphragmatic VAX may not be a reliable and reproducible method to produce animal models of GI diseases induced by long-term functional reduction of GI motility in humans.

It has been reported that subdiaphragmatic VAX in guinea pigs increased daily food intake and weight gain, whereas the reverse occurred in rats[23,24], and a pathological depletion of thyroid hormones causes a reduction in normal weight gain in rats[14]. These findings are partially in accord with our findings, indicating that unilateral TPX plus VAX significantly decreases body weight gain, but not daily dietary intake. We carefully consider that these may be related to the experimental methods used, which may differently regulate the physiological functions of gut including gastric secretion and bowel motility, resulting in different magnitudes of food intake and weight gain. This is supported by previous reports[1,25],

suggesting that alteration of food intake and weight gain following bilateral TPX and subdiaphragmatic VAX is absolutely dependent on the ability of the GI tract to digest food.

The delayed gastric emptying in some patients with hypothyroidism is likely to result from reduced muscle contractility of the antrum[26], and vagal nerves are involved in the regulation of contractile patterns during gastric phase Ⅲ[27]. In rats, chronic hypothyroidism or hyperthyroidism alters the expression patterns of cholinergic muscarinic receptors distributed in the ileal muscles[28], and administration of carbachol obviously increases ileal transit decline by vagal denervation[8]. These suggestions are well in accord with our results, indicating that unilateral TPX plus VAX markedly delays gastric emptying and small bowel transit. Although this study did not evaluate the cellular mechanisms implicated in the unilateral TPX plus VAX-induced reduction of upper GI motility, the results found in this study likely suggest that unilateral TPX plus VAX may be a reliable and reproducible method to produce a rat model characterized by a long-term reduction in gastric emptying and small bowel transit.

A previous report[8] suggested that colonic transit was delayed soon after subdiaphragmatic VAX, but returned to essentially normal levels at a later time. We also observed a similar effect of subdiaphragmatic VAX on defecatory function. Importantly, a reduction in defecatory function was maintained for 7 d when unilateral TPX and subdiaphragmatic VAX were sequentially applied to rats. These results clearly indicated that unilateral TPX plus VAX obviously reduces the colonic motor activity which propels the luminal contents, and the reduced propulsive activity of the upper and lower GI tract is maintained for long time periods compared with subdiaphragmatic VAX alone. Moreover, it is considered that a significant decrease in the daily numbers of fecal pellets is possibly caused by a reduction in the defecatory function of the colon, and this likely reflects the reduction in motor function of colonic smooth muscle observed in patients with constipation and defecatory disorders[25].

It has been well known that ACh[29] is the major excitatory neurotransmitter, and nitric oxide[30] is the major non-adrenergic, non-cholinergic inhibitory neurotransmitter in the regulation of colonic motility. Therefore, to assess the alteration in excitatory and inhibitory neurotransmitter systems by unilateral TPX plus VAX, we examined the contractile responses of colonic strips to ACh and L-NAME. In comparison with colonic strips of normal rats, the colonic strips prepared from unilateral TPX plus VAX rats exhibited a markedly impaired contractile response to ACh, but the contractile response to L-NAME was not significantly altered. We also found that unilateral TPX plus VAX did not significantly affect the contractile properties of adrenergic and purinergic inhibitory mechanisms (data not shown). These results are possibly suggestive of cholinergic neuronal remodeling during a partial depletion of thyroparathyroid hormones and disruption of subdiaphragmatic vagal nerves, resulting in functional reduction in colonic motility leading to pathological conditions such as constipation. Findings comparable with our results have previously been reported, suggesting that depletion of thyroparathyroid hormones reduces GI motility through a reduction in excitatory neurotransmitter activity[6]. Therefore, the results found in this study suggest that, in addition to dysfunction of vagal nerves regulating GI motility, the reduced colonic contractility may result from enteric excitatory neuronal loss associated with a decrease in thyroparathyroid hormones. However, further studies are needed to investigate the detailed cellular mechanisms involved in the unilateral TPX plus VAX-induced reduction of GI motility.

Taken together, sequential application of unilateral TPX and subdiaphragmatic VAX to rats may be a reliable method to produce a model of delayed gastric emptying and defecatory disorders in rats. In comparison with bilateral TPX or subdiaphragmatic VAX alone, TPX plus VAX showed valuable advantages, including no animal deaths and long-term persistence of reduced GI motility. Rats sequentially undergoing unilateral TPX and subdiaphragmatic VAX may be taken into consideration as a putative animal model for disorders of GI motility such as dyspepsia and constipation.

COMMENTS

Background

The prevalence of dyspepsia and constipation in humans continues to increase throughout the world. Although attenuation of gastric and colonic motility is widely considered as the primary causal factors for the occurrence of dyspepsia and constipation, respectively, there are still many questions to be answered, and a useful animal model to evaluate the pathophysiological mechanisms responsible for the reduction of gut motility remains to be developed.

Research frontiers

Thyroid diseases affect gastrointestinal (GI) functions including disturbance of gut motility, and the most common GI complaints in patients with hypothyroidism are nausea, vomiting, constipation and abdominal pain. The vagal nerves are extensively distributed in the digestive tracts from the upper esophageal sphincter muscles to the transverse colon in humans, and the extensive distribution of vagal nerves to the gut provides a key bi-directional link with the brain, which regulates GI behavior such as food intake, gastric accommodation and muscle contractility.

Innovations and breakthroughs

It has been demonstrated in our study using rats that complete depletion of thyroparathyroid hormones by thyroparathyroidectomy caused an extremely high fatality rate, and the disturbance of vagal activity by thyroparathyroidectomy produced a transient, less than 3 d, reduction of gastrointestinal motility. However, in comparison with thyroparathyroidectomy or vagotomy alone, application of both unilateral thyroparathyroidectomy and subdiaphragmatic vagotomy to rats had several advantages, including no deaths of the animals and a long-term, at least 7 d, reduction in gut motility. In the present study, the authors suggest for the first time that both unilateral thyroparathyroidectomy and vagotomy may be reliably applied to produce a rat model of GI disorders characterized by long-term reduction in GI motility.

Applications

Rats subject to both unilateral thyroparathyroidectomy and subdiaphragmatic thyroparathyroidectomy may provide an animal model of dyspepsia and constipation, and may be usefully used in studies of new drugs to reverse the reduced motility of the GI tract.

Terminology

Dyspepsia: A condition of impaired gastric digestive function associated with gastritis and gastroesophageal reflux. The common symptoms of dyspepsia are upper abdominal fullness, bloating, nausea, heartburn or feeling full earlier than expected.

Peer review

The authors have attempted to develop a novel animal model for reducing gastrointestinal motility in aid of studies examining dyspepsia and constipation. This is a straightforward study that addresses an important issue.

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BRIEF ARTICLE

Does immunohistochemical staining have a clinical impact in early gastric cancer conducted endoscopic submucosal dissection?

Seong Ran Jeon, Joo Young Cho, Gene Hyun Bok, Tae Hee Lee, Hyun Gun Kim, Won Young Cho, So Young Jin, Yeon Soo Kim

Seong Ran Jeon, Joo Young Cho, Gene Hyun Bok, Tae Hee Lee, Hyun Gun Kim, Won Young Cho, Institute for Digestive Research, Digestive Disease Center, Soonchunhyang University College of Medicine, Seoul 140-743, South Korea
So Young Jin, Department of Pathology, Soonchunhyang University College of Medicine, Seoul 140-743, South Korea
Yeon Soo Kim, Department of Internal Medicine, Daejeon St. Mary's Hospital, The Catholic University of Korea School of Medicine, Daejeon 301-723, South Korea
Author contributions: Jeon SR drafted the article, analysis and interpreted of the data; Bok GH, Lee TH, Kim HG, Cho WY, and Kim YS were responsible for analysis and interpretation of the data; Jin SY reviewed data analysis and corrected research design; Cho JY made the critical revision of the article for important intellectual content and final approval of the article.
Correspondence to: Joo Young Cho, Professor, MD, PhD, Institute for Digestive Research, Digestive Disease Center, Soonchunhyang University College of Medicine, 657 Hannam-Dong, Yongsan-Gu, Seoul 140-743, South Korea. cjy6695@dreamwiz.com
Telephone: + 82-2-7099202 Fax: +82-2-7099696
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Abstract

AIM: To evaluate clinicopathologic parameters and the clinical significance related lymphovascular invasion (LVI) by immunohistochemical staining (IHCS) in endoscopic submucosal dissection (ESD).

METHODS: Between May 2005 and May 2010, a total of 348 lesions from 321 patients (mean age 63 ± 10 years, men 74.6%) with early gastric cancer (EGC) who met indication criteria after ESD were analyzed retrospectively. The 348 lesions were divided into the absolute (n = 100, differentiated mucosal cancer without ulcer ≤ 20 mm) and expanded (n = 248) indication groups after ESD. The 248 lesions were divided into four subgroups according to the expanded ESD indication. The presence of LVI was determined by factor Ⅷ-related antigen and D2-40 assessment. We compared LVI IHCS-negative group with LVI IHCS-positive in each group.

RESULTS: LVI by hematoxylin-eosin staining (HES) and IHCS were all negative in the absolute group, while was observed in only the expanded groups. The positive rate of LVI by IHCS was higher than that of LVI by HES (n = 1, 0.4% *vs* n = 11, 4.4%, P = 0.044). LVI IHCS-positivity was observed when the cancer invaded to the mucosa 3 (M3) or submucosa 1 (SM1) levels, with a predominance of 63.6% in the subgroup that included only SM1 cancer (P < 0.01). In a univariate analysis, M3 or SM1 invasion by the tumor was significantly associated with a higher rate of LVI by IHCS, but no factor was significant in a multivariate analysis. There were no cases of tumor recurrence or metastasis during the median 26 mo follow-up.

CONCLUSION: EGCs of the absolute group are immunohistochemically stable. The presence of LVI may be carefully examined by IHCS in an ESD expanded indication group with an invasion depth of M3 or greater.

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Key words: Gastric cancer; Endoscopic submucosal dissection; Immunohistochemical staining; Lymphovascular invasion; Depth

Peer reviewers: Magdy El-Salhy, Professor, Department of Gastroenterology, School of Medicine, Stord Helse-Fonna Hospital, 5409 Stord, Norway; Dr. Cuneyt Kayaalp, Professor, Depatment of General Surgery, Inonu University, 44315 Malatya, Turkey

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INTRODUCTION

The incidence of early gastric cancer (EGC) has increased in Asia, and particularly in South Korea and Japan[1]. Endoscopic submucosal dissection (ESD) can be used for *en bloc* and complete resection (CR), and is widely applied for the curative treatment of EGC[2-4]. Studies comparing the long-term results of EGC treatment have demonstrated no significant increase in the five-year survival rate of patients following ESD[2,5,6]. However, these techniques are necessary for proper diagnosis of tumor histology to check whether the tumor has been completely removed or has recurred and to predict the possibility of lymph node (LN) metastasis, thereby determining the need for additional surgery.

Assessment of lymphovascular invasion (LVI) is usually performed on the basis of conventional hematoxylin-eosin staining (HES), sometimes lacks objectivity possibly because of the inability to distinguish lymphatics from blood vessels[7]. Although the clinical significance of molecular-based methods using immunohistochemical staining (IHCS) of resected tissues is controversial, IHCS has been used to evaluate the presence of LVI, which a risk factor for LN metastasis[8-10]. Thus, we evaluated possible correlations between IHCS and other clinicopathologic parameters in ESD specimens and the clinical significance of IHCS.

MATERIALS AND METHODS

Study design and patients

Data were retrospectively collected from patients who underwent ESD at a single, tertiary-care, academic medical center. Between May 2005 and May 2010, a total of 348 lesions (321 patients) that met the absolute and expanded indication criteria after ESD were included. This study was approved by our hospital ethics committee and institutional review board. Written informed consent was obtained from all patients prior to the procedure.

Pre-ESD evaluation

The pre-ESD assessment of tumor size and depth of invasion was performed by conventional endoscopy and endoscopic ultrasonography. Contrast-enhanced computed tomography was also performed in all patients before treatment to exclude involvement of lymph nodes or distant metastasis.

ESD and pathologic analysis

ESD was performed as reported previously[11,12]. All ESD specimens were examined by an experienced pathologist (Jin SY). Resected specimens were fixed in 10% formalin and sectioned serially in 2-mm intervals, then subjected to histological analysis. All slices were embedded in a paraffin block. The section margin, depth of invasion, and LVI were observed carefully. A one-piece resection was defined as an *en bloc* resection. The definition of CR was: (1) *en bloc* resection or complete reconstruction in two piecemeal resection cases; (2) being free of cancer at both the vertical (VM) and lateral margins (LM); (3) no submucosal invasion deeper than 500 μm from the muscularis mucosa; and (4) no evidence of vascular or lymphatic invasion. Incomplete resection was defined as resections that did not meet the curative criteria. Incompletely resected lesions confined to the mucosa with positive LM were scheduled for an additional ESD procedure, argon plasma coagulation, or surgery.

In pathology specimens obtained by ESD, the absolute indication criteria for ESD was intramucosal differentiated adenocarcinoma without ulceration $\leqslant$ 20 mm in diameter. The expanded indication criteria for ESD were: (1) intramucosal differentiated adenocarcinoma without ulceration > 20 mm in diameter; (2) intramucosal differentiated adenocarcinoma with ulceration $\leqslant$ 30 mm in diameter; (3) submucosa 1 (SM1) differentiated adenocarcinoma $\leqslant$ 30 mm in diameter; and (4) intramucosal undifferentiated adenocarcinoma without ulceration $\leqslant$ 20 mm in diameter. In this order, the expanded indication group was divided into four subgroups (groups 1, 2, 3 and 4).

Immunohistochemical staining

Serial sections (4 μm-thick) were deparaffinized in xylene. Samples were rehydrated in a graded alcohol series, and heated for 10 min in a microwave oven. This procedure was repeated twice after immersion in a citrate buffer solution to increase the reproducibility of endogenous antigens. To detect endogenous peroxidase, the samples were placed in 3% hydrogen peroxide solution for 10 min and rinsed with phosphate-buffered saline (PBS). Nonspecific binding sites were blocked by incubation in 3% normal non-immune serum for 10 min. Then the slides were washed with PBS and incubated with biotinylated polyvalent secondary antibody at room temperature for 15 min. After washing with PBS, the slides were incubated with streptavidine peroxidase for 15 min and washed a second time. Immunoreactive proteins were visualized by incubation with amine-ethyl carbazole for 3 to 5 min. The samples were counterstained with Mayer's HE and mounted using crystal mount (Biomeda, Foster City, CA, United States).

Primary antibodies specific for the mucin and gastric phenotypic markers, MUC5AC (CLH2, Novocastra Lab. Ltd, Newcastle, United Kingdom) and MUC6 (CLH5, Novocastra Lab. Ltd, Newcastle, United Kingdom), the intestinal phenotypic marker MUC2 (CCP58, Biogenx, San Ramon, CA, United States), and CD10 (56C6, Signet Lab. Oakland, CA, United States) were used. Samples that were positive for MUC5AC or MUC6 were classi-

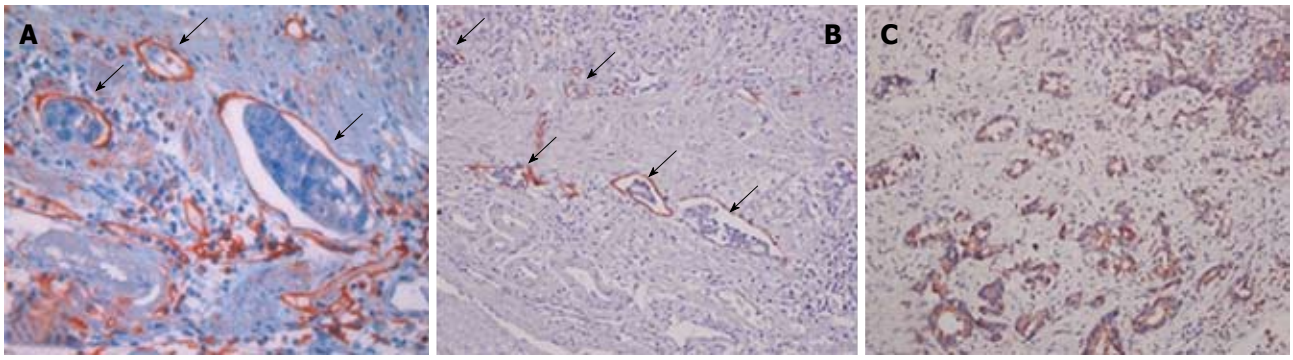


Figure 1 Immunohistochemical findings of early gastric cancers. A: Three foci of vascular invasion of tumor cells (arrows) are seen (factor Ⅷ related antigen, × 400); B: Multiple foci of lymphatic invasion (arrows) are found in the both mucosa and submucosa (D2-40, ×200); C: Positive staining is noted in the cytoplasm of neoplastic glands (vascular endothelial growth factor, ×200).

fied as having a gastric phenotype, and those positive for MUC2 or CD10 were classified as having an intestinal phenotype. Samples that were positive for at least one marker of both phenotypes were considered to have a mixed phenotype, and samples showing no marker immunoreactivity were considered to be an unclassified phenotype.

Lymphovascular-related markers and IHCS analysis

For the vascular endothelial marker, primary antibodies against factor Ⅷ-related antigen (FⅧRAg, 1:3000; DAKO, Carpenteria, CA, United States) were used. For the lymphatic endothelial marker, primary antibodies against D2-40 (1:200; LI-D, Covance Inc., Cumberland, VA, United States) were used. Primary antibodies against vascular endothelial growth factor (VEGF) (1:50; SC-152, Santa Cruz Biotechnology, Santa Cruz, CA, United States) were also applied. Vascular or LVI was indicated by clusters of tumor cell in vessels or lymphatic vasculature, the endothelia of which stained positive for FⅧRAg or D2-40, respectively. VEGF was considered positive when anti-VEGF antibodies stained the tumor cytoplasm. High microvessel density (MVD) and lymphovascular density (LVD) were detected by increased intra-tumor staining of factor Ⅷ and D2-40 (Figure 1).

Post-ESD follow-up

Endoscopic follow-up examinations were performed routinely at 1, 3, 6 and 12 mo, and then annually after ESD to assess the completeness of resection and to detect metachronous lesions. During the follow-up period, biopsy samples were taken from the ESD ulcer scar or other suspicious mucosal abnormalities. If a residual lesion or metachronous lesion was noted, additional ESD or surgery was planned.

Statistical analysis

Statistical analyses were performed using the SPSS software (version 15.0; SPSS, Chicago, IL, United States). Categorical variables were analyzed by the Chi-square test or Fisher's exact test and non-categorical variables were analyzed by the *t* test to verify the relationship between IHCS and other clinicopathological parameters. Non-categorical variables were compared among the four groups using an analysis of variance test. A multivariate analysis was conducted using a logistic regression method. *P* values $\leqslant$ 0.05 indicated statistical significance.

RESULTS

Endoscopic and pathologic characteristics

In the absolute group, the mean age of the 100 patients (100 lesions) was 62.2 years (range: 33-86 years) and included 70 men (70%). In the expanded group, the mean age of the 221 patients (248 lesions) was 62.9 years (range: 33-91 years) and included 189 men (76.2%). The dominant tumor location in both groups was the lower third of the stomach. Macroscopically, the flat/depressed type was most common (n = 131, 52.8%), and ulcers were observed in 64.9% (n = 161) of patients in the expanded group.

Of 248 lesions in the expanded group, 238 (96%) were differentiated and 10 (4%) were poorly differentiated adenocarcinoma. Twenty-eight lesions (11.3%) were SM1 cancer. The mean tumor size was 21.2 mm (range: 3-86 mm). Twenty-four (9.7%) were LM-positive two (0.8%) were VM-positive. The mixed mucin phenotype (n = 90, 36.3%) was predominant. A total of 72 (29%) were VEGF positive and 59 (23.8%) exhibited a high MVD/LVD. The CR rate by IHCS was significantly lower than that by HES (84.3% *vs* 88.3%, $P < 0.01$). The endoscopic and histopathologic characteristics of the lesions in the absolute and expanded groups are summarized in Table 1.

Subgroup analysis in the expanded indication

As mentioned above, the 248 lesions were divided into four groups according to the expanded ESD indication. Groups 1, 2, 3 and 4 contained 71 (28.6%), 140 (56.4%), 27 (10.8%) and 10 (4.7%) lesions, respectively. LVI HES-positivity was also defined as when LVI was positive by HE staining, and LVI IHCS-positivity was also defined as when LVI was positive by IHCS. Of a number of clinicopathological factors, only LVI IHCS-positivity showed significant differences between subgroups. LVI

Table 1 Endoscopic and pathologic characteristics of endoscopic submucosal dissection groups

Groups	Absolute (n = 100)	Expanded (n = 248)	P value
Age (yr), mean (range)	62.2 (33-86)	62.9 (33-91)	0.589
Male (%)	70 (70)	189 (76.2)	0.277
Location (%)			0.183
Upper	3 (3)	21 (8.5)	
Middle	31 (31)	69 (27.8)	
Lower	66 (66)	158 (63.7)	
Macroscopic type (%)			< 0.001
Elevated	59 (59)	56 (22.6)	
Flat/depressed	36 (36)	131 (52.8)	
Mixed	5 (5)	61 (24.6)	
Endoscopic ulcer (%)			< 0.001
Present	0 (0)	161 (64.9)	
Absent	100 (100)	87 (35.1)	
Resection (%)			0.053
En-bloc	100 (100)	234 (94.4)	
Complete reconstruction	0 (0)	8 (3.2)	
⩾ 3 piecemeal	0 (0)	6 (2.4)	
Histology (%)			0.069
Differentiated	100 (100)	238 (96)	
Undifferentiated	0 (0)	10 (4)	
Depth (%)			< 0.001
M2	59 (59)	89 (35.9)	
M3	41 (41)	131 (52.8)	
SM1	0 (0)	28 (11.3)	
Tumor size (mean, range)	11.4 (2-20)	21.2 (3-86)	< 0.001
⩽ 20 mm (%)	100 (100)	131 (52.8)	
> 20 mm (%)	0 (0)	117 (47.2)	
Lateral margin (%)			< 0.001
Positive	0 (0)	24 (9.7)	
Negative	100 (100)	224 (90.3)	
Vertical margin (%)			0.500
Positive	0 (0)	2 (0.8)	
Negative	100 (100)	246 (99.2)	
Phenotype (%)			0.312
Gastric	24 (24)	66 (26.6)	
Intestinal	31 (31)	92 (37)	
Mixed	45 (45)	90 (36.3)	
VEGF (%)			0.096
Positive	79 (79)	72 (29)	
Negative	21 (21)	176 (71)	
High MVD/LVD (%)			0.116
Positive	16 (16)	59 (23.8)	
Negative	84 (84)	189 (76.2)	
LVI HES			0.713
Positive	0 (0)	1 (0.4)	
Negative	100 (100)	247 (99.6)	
LVI IHCS			0.038
Positive	0 (0)	11 (4.4)	
Negative	100 (100)	237 (95.6)	

M: Mucosa; SM: Submucosa; VEGF: Vascular endothelial growth factor; MVD: Microvessel density; LVD: Lymphatic vessel density; LVI: Lymphovascular invasion; HES: Hematoxylin-eosin staining; IHCS: Immunohistochemical staining.

HES-positivity was observed only in group 4, which included undifferentiated carcinoma. While LVI IHCS-positivity was observed in all subgroups, there was a predominance of 63.6% (7/11) in group 3, which included only SM1 cancer ($P < 0.001$) (Table 2).

Comparison of LVI in the immunohistochemically negative and positive groups in the expanded indication

LVI HES-positivity was confirmed by LVI IHCS; however, of 11 lesions identified as LVI IHCS-positive, only one was also LVI HES-positive (100% *vs* 9.1%, $P = 0.044$) (Table 3).

The association between LVI IHCS and various clinicopathological factors and IHCS markers was analyzed (Table 4). In this statistical analysis, we combined the three tumor location and depth of invasion categories into two "upper + corpus" and "lower + angle", and "mucosa 2 (M2)" and "M3 + SM1", respectively. Phenotype was categorized into intestinal and non-intestinal type. LVI IHCS-positivity was detected in 4.4% (11/248). In a univariate analysis, M3 or SM1 invasion of the tumor, and the presence of VEGF and a high LVD/MVD were factors significantly associated with a higher rate of LVI by IHCS. However, there were no significant as-

Table 2 Subgroup analysis in the expanded indication groups

	Group 1 (n = 71)	Group 2 (n = 140)	Group 3 (n = 27)	Group 4 (n = 10)	P value
Age > 60 (yr, %)	44 (62)	82 (58.6)	21 (77.8)	6 (60)	0.502
Male (%)	56 (78.9)	104 (74.3)	24 (88.9)	5 (50)	0.455
Location (%)					0.730
Upper + corpus	19 (26.8)	15 (10.7)	11 (40.7)	1 (10)	
Lower + angle	52 (73.2)	125 (89.3)	16 (59.3)	9 (90)	
Macroscopic type (%)					< 0.001
Elevated	45 (63.4)	4 (2.9)	4 (14.8)	3 (30)	
Flat/depressed	26 (36.6)	136 (97.1)	23 (85.2)	7 (70)	
Endoscopic ulcer (%)					< 0.001
Present	0 (0)	140 (100)	20 (74.1)	1 (10)	
Absent	71 (100)	0 (0)	7 (25.9)	9 (90)	
Resection, piece (%)					0.177
⩽ 2	68 (95.8)	137 (97.9)	27 (100)	10 (100)	
⩾ 3	3 (4.2)	3 (2.1)	0 (0)	0 (0)	
Depth (%)					0.003
M2	33 (46.5)	52 (37.1)	0 (0)	4 (40)	
M3 + SM1	38 (53.5)	88 (62.9)	27 (100)	6 (60)	
Tumor size, mm (%)					< 0.001
⩽ 20	0 (0)	105 (75)	16 (59.3)	10 (100)	
> 20	71(100)	35 (25)	11 (40.7)	0 (0)	
Lateral margin (%)					0.091
Positive	10 (14.1)	11 (7.9)	1 (3.7)	2 (20)	
Negative	61 (85.9)	129 (92.1)	26 (96.3)	8 (80)	
Vertical margin (%)					0.170
Positive	2 (2.8)	0 (0)	0 (0)	0 (0)	
Negative	69 (97.2)	140 (100)	27 (100)	10 (100)	
Phenotype (%)					0.107
Intestinal	31 (43.7)	50 (35.7)	9 (33.3)	2 (20)	
Non-intestinal	40 (56.3)	90 (64.3)	18 (66.7)	8 (80)	
VEGF (%)					0.003
Positive	17 (23.9)	34 (24.3)	16 (59.3)	5 (50)	
Negative	54 (76.1)	106 (75.7)	11 (40.7)	5 (50)	
High LVD/MVD (%)					< 0.001
Positive	11 (15.5)	26 (18.6)	16 (59.3)	6 (60)	
Negative	60 (84.5)	114 (81.4)	11 (40.7)	4 (40)	
LVI HES					0.005
Positive	0 (0)	0 (0)	0 (0)	1 (10)	
Negative	71 (100)	140 (100)	27 (100)	9 (90)	
LVI IHCS					< 0.001
Positive	1 (1.4)	1 (0.7)	7 (25.9)	2 (20)	
Negative	70 (98.6)	139 (99.3)	20 (74.1)	8 (80)	

M: Mucosa; SM: Submucosa; VEGF: Vascular endothelial growth factor; MVD: Microvessel density; LVD: Lymphatic vessel density; LVI: Lymphovascular invasion; HES: Hematoxylin-eosin staining; IHCS: Immunohistochemical staining.

Table 3 Comparison of lymphovascular invasion hematoxylin-eosin staining and lymphovascular invasion immunohistochemical staining groups

ESD indication			LVI IHCS		Total	*P* value
			Negative	Positive		
Absolute	LVI HES	Negative	100	0	100	
		Positive	0	0	0	
	Total		100	0	100	
Expanded	LVI HES	Negative	237	10	247	0.044
		Positive	0	1	1 (0.4%)	
	Total		237	11 (4.4%)	248	

ESD: Endoscopic submucosal dissection; LVI: Lymphovascular invasion; HES: Hematoxylin-eosin staining; IHCS: Immunohistochemical staining.

sociations between LVI by IHCS and age, sex, location, macroscopic type, endoscopic ulcer, resection pieces, histology, tumor size, lateral margin, vertical margin, or phenotype. In a multivariate analysis, there were no significant associations between LVI in IHCS and these factors (Table 4).

Follow-up

Of the 321 patients who underwent ESD, 5 (1.6%) required additional surgery (Table 5). The reasons for additional surgery were an insufficient gap between the tumor and LM or VM in four patients and patient need in one. No lesions exhibited LN metastasis. Two of the five patients had a residual tumor in the gastric wall. There were no cases of tumor recurrence or metastasis after ESD, including those patients who underwent additional surgery, during the median 26 mo follow-up.

DISCUSSION

LVI and increased LVD detected by IHCS are associated with LN metastasis in gastric cancer[13-18], and are correlated with an unfavorable prognosis in other types of cancer[19-23]. However, detection of LVI by HES is difficult for several reasons. Primarily, the distinction between blood vessels and lymphatic vessels is subjective. Furthermore, artifacts or artificial spaces formed during sample preparation may mimic the tubular structure of vasculature[7]. Antibodies to FⅧRAg help to distinguish true vascular endothelial cells therefore increasing the sensitivity of detection of vascular invasion[24,25] whereas the monoclonal antibody D2-40 specific for lymphatic endothelial cells enhances detection of lymphatic invasion[26-28]. In a previous study using post-operative specimens to diagnose lymphatic invasion, D2-40 was more sensitive for detection of lymphatic invasion (positive rate 44% *vs* 27% for D2-40 *vs* HE)[27]. Thus, we evaluated the clinical significance of IHCS, using for example FⅧRAg and D2-40, in patients with the expanded indication criteria after ESD. To the best of our knowledge, no study has evaluated the possible correlations between IHCS and other clinicopathological parameters in the expanded indication of ESD.

Table 4 Comparison of lymphovascular invasion by immunohistochemical stain negative and positive group

	LVI IHCS		*P* value
	Negative (*n* = 237)	Positive (*n* = 11)	
Age, yr (%)	62.6 ± 9.9	69.4 ± 10.2	0.054
≤ 60	92 (38.8)	3 (27.3)	
> 60	145 (61.2)	8 (72.7)	
Male (%)	182 (76.8)	7 (63.6)	0.297
Location (%)			0.667
Upper + corpus	44 (18.6)	2 (18.2)	
Lower + angle	193 (81.4)	9 (81.8)	
Macroscopic type (%)			0.715
Elevated	53 (22.4)	3 (27.3)	
Flat/depressed	184 (77.6)	8 (72.7)	
Endoscopic ulcer (%)			0.524
Present	155 (65.4)	6 (54.5)	
Absent	82 (34.6)	5 (45.5)	
Resection, piece (%)			0.760
≤ 2	231 (97.5)	11 (100)	
≥ 3	6 (2.5)	0 (0.0)	
Histology (%)			0.066
Differentiated	229 (96.6)	9 (81.8)	
Undifferentiated	8 (3.4)	2 (18.2)	
Depth (%)			0.009
M2	89 (37.6)	0 (0)	
M3 + SM1	148 (62.4)	11 (100)	
Tumor size, mm (%)	21.2 ± 12.8	21.2 ± 16.7	0.986
≤ 20	125 (52.7)	6 (54.5)	
> 20	112 (47.3)	5 (45.5)	
Lateral margin (%)			0.289
Positive	22 (9.3)	2 (18.2)	
Negative	215 (90.7)	9 (81.8)	
Vertical margin (%)			0.913
Positive	2 (0.8)	0 (0)	
Negative	235 (99.2)	11 (100)	
Phenotype (%)			0.751
Intestinal	89 (37.6)	3 (27.3)	
Non-intestinal	148 (62.4)	8 (72.7)	
VEGF (%)			< 0.001
Positive	62 (26.2)	10 (90.9)	
Negative	175 (73.8)	1 (9.1)	
High LVD/MVD (%)			< 0.001
Positive	48 (20.3)	11 (100)	
Negative	189 (79.7)	0 (0.0)	

IHCS: Immunohistochemical staining; M: Mucosa; SM: Submucosa; VEGF: Vascular endothelial growth factor; LVD: Lymphatic vessel density, MVD: Microvessel density.

In this study, 11 lesions in the only expanded indication group, but not the absolute indication group, displayed LVI-positivity. This is likely because lymphatics existed only in the deep mucosa and abundant microvessels were found in the muscularis mucosa[27]. Differentiated mucosal cancer, which belongs to the absolute indication group was LVI-negative. Because the EGCs of the absolute indication group were immunohistochemically stable, the clinical implication of IHCS in this group is insignificant.

In the expanded indication, comparing the LVI IHCS-negative and LVI IHCS-positive groups, M3 or SM1 invasion of the tumor and the presence of VEGF and a high LVD/MVD were factors significantly associated with a higher rate of LVI by IHCS. However,

Table 5 Clinicopathological characteristics of the five patients who underwent surgical resection after endoscopic submucosal dissection

Sex/age (yr)	Type	Site	Ulcer	Pathology	Size (mm)	LM/VM	Depth	LVI HES	LVI IHCS	Residual tumor	LN
Female/67	Ⅱc	Lower	-	MD	30	-/+	M	-	-	+	-
Male/74	Ⅲ	Middle	+	WD	14	+/-	M	+	+	-	-
Female/64	Ⅲ	Middle	+	MD	19	+/-	M	-	-	-	-
Female/54	Ⅰ	Middle	-	MD	68	+/-	M	-	+	+	-
Male/35	Ⅰ	Middle	-	MD	22	-/-	M	-	-	-	-

LM: Lateral margin; VM: Vertical margin; LVI: Lymphovascular invasion; HES: Hematoxylin-eosin staining; IHCS: Immunohistochemical staining; LN: Lymph node; MD: Moderately differentiated; WD: Well differentiated; M: Mucosa.

there were no significant associations between LVI by IHCS and these factors in a multivariate analysis. This may be because, first, the number of patients in the LVI IHCS-positive group was too small compared to the LVI IHCS-negative group. Second, although the specimens were examined by a highly experienced pathologist, the interpretation of the IHCS results was not always accurate. Nonetheless, we must not overlook the fact that LVI-positivity was observed when the cancer invaded the M3 or SM1 layer in the LVI IHCS-positive group. Resected specimens examined by HES showed an LVI false-negative rate of 4% and a false-positive rate of 0%. The positive rate of LVI by IHCS was significantly higher than that of LVI by HES (4% *vs* 0.4%, $P = 0.044$). In other words, 10 tumors, which had been diagnosed as no LVI by HES, were correctly diagnosed as having LVI after IHCS. Only 1 of 11 (9%) LVI, confirmed by IHCS, was correctly diagnosed by HES. These results suggest that LVI cannot be predicted by HES alone and also that IHCS is somewhat more helpful than HES for identifying LVI. Therefore, the presence of LVI should be carefully examined by IHCS when invasion with a depth of M3 or greater has been detected in an expanded indication.

The major limitations of this study are its retrospective design, the relatively short follow-up period and the limited number of patients with LVI by IHCS. Because the number of LVI by IHCS was small and additional surgical treatment was not provided to all patients who were LVI-positivity by IHCS, we were unable to explain the association between LVI IHCS-positivity and LN metastasis. According to the Japanese classification of gastric carcinoma[29], resected specimens were cut thinner (*vs* surgical specimens; 2 mm *vs* 4 mm). Therefore, the quality of pathologic interpretation of resected specimens was better than that of surgical specimens. We found no case of metastasis in the LVI IHCS-positive group during the median 26 mo follow-up. However, because a previous study reported that LN metastasis was confirmed in 89% of D2-40 positive cases, compared to 41% of suspected cases of metastasis based on HES[14], a future large and long-term follow-up study is needed to assess via IHCS the relationship of LVI with LN metastasis, tumor recurrence, and patient survival.

Examination by IHCS may be required because LVI can be undetected in an ESD expanded indication group with an invasion depth of M3 or greater if we rely only on HES.

COMMENTS

Background

Lymphovascular invasion (LVI) detected by immunohistochemical staining (IHCS) is associated with lymph node (LN) metastasis in gastric cancer. The clinical significance of IHCS in early gastric cancer (EGC) in association with endoscopic submucosal dissection (ESD) is controversial. No study has evaluated the possible correlations between IHCS and other clinicopathological parameters in ESD specimens.

Research frontiers

Relationships between LN micrometastasis and clinicopathological findings based on D2-40 and factor Ⅷ-related antigen (FⅧRA) IHCS in gastric cancer have been reported. Several studies have reported that IHCS is more sensitive for detection of LVI.

Innovations and breakthroughs

LVI by hematoxylin-eosin stain (HES) and IHCS were all negative in an ESD absolute group; this group was immunohistochemically stable. However, in an ESD expanded group, the LVI IHCS-positive rate was significantly higher than that of LVI by HES. LVI IHCS-positivity was observed when the cancer invaded the mucosa 3 (M3) or submucosa 1 (SM1) levels. Therefore, the presence of LVI should be carefully examined by IHCS in an expanded group with an invasion depth of M3 or SM1.

Applications

In an ESD expanded indication group with an invasion depth of M3 or greater, IHCS should be used because LVI-positivity can remain undetected by HES. However, a large-scale prospective study is required.

Terminology

ESD, a method that can be used for *en bloc* and complete resection of EGC, is widely applied for the curative treatment of EGC. IHCS is widely used in the diagnosis of abnormal cells using specific molecular markers, such as D2-40 and FⅧRA.

Peer review

Because the number of LVIs detected by IHCS was small and additional surgical treatment was not provided to all patients who were LVI-positive by IHCS, it was unable to explain the association between LVI IHCS-positivity and LN metastasis. However, this study implies the clinical significance of IHCS in an ESD expanded indication group, and reports a useful methodology.

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BRIEF ARTICLE

Spectral analysis of bowel sounds in intestinal obstruction using an electronic stethoscope

Siok Siong Ching, Yih Kai Tan

Siok Siong Ching, Yih Kai Tan, Department of Surgery, Changi General Hospital, Singapore 529889, Singapore
Author contributions: Ching SS conceived and designed the study, provided supporting material, acquired the data, performed the statistical analysis, interpreted the data and wrote the manuscript; Tan YK supervised the study and critically revised the manuscript.
Correspondence to: Siok Siong Ching, MRCS, MD, Department of Surgery, Changi General Hospital, 2 Simei Street 3, Singapore 529889, Singapore. ching_ss@hotmail.com
Telephone: +65-97729648 Fax: +65-62601709
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Abstract

AIM: To determine the value of bowel sounds analysis using an electronic stethoscope to support a clinical diagnosis of intestinal obstruction.

METHODS: Subjects were patients who presented with a diagnosis of possible intestinal obstruction based on symptoms, signs, and radiological findings. A 3M™ Littmann® Model 4100 electronic stethoscope was used in this study. With the patients lying supine, six 8-second recordings of bowel sounds were taken from each patient from the lower abdomen. The recordings were analysed for sound duration, sound-to-sound interval, dominant frequency, and peak frequency. Clinical and radiological data were reviewed and the patients were classified as having either acute, subacute, or no bowel obstruction. Comparison of bowel sound characteristics was made between these subgroups of patients. In the presence of an obstruction, the site of obstruction was identified and bowel calibre was also measured to correlate with bowel sounds.

RESULTS: A total of 71 patients were studied during the period July 2009 to January 2011. Forty patients had acute bowel obstruction (27 small bowel obstruction and 13 large bowel obstruction), 11 had subacute bowel obstruction (eight in the small bowel and three in large bowel) and 20 had no bowel obstruction (diagnoses of other conditions were made). Twenty-five patients received surgical intervention (35.2%) during the same admission for acute abdominal conditions. A total of 426 recordings were made and 420 recordings were used for analysis. There was no significant difference in sound-to-sound interval, dominant frequency, and peak frequency among patients with acute bowel obstruction, subacute bowel obstruction, and no bowel obstruction. In acute large bowel obstruction, the sound duration was significantly longer (median 0.81 s *vs* 0.55 s, $P = 0.021$) and the dominant frequency was significantly higher (median 440 Hz *vs* 288 Hz, $P = 0.003$) when compared to acute small bowel obstruction. No significant difference was seen between acute large bowel obstruction and large bowel pseudo-obstruction. For patients with small bowel obstruction, the sound-to-sound interval was significantly longer in those who subsequently underwent surgery compared with those treated non-operatively (median 1.29 s *vs* 0.63 s, $P < 0.001$). There was no correlation between bowel calibre and bowel sound characteristics in both acute small bowel obstruction and acute large bowel obstruction.

CONCLUSION: Auscultation of bowel sounds is non-specific for diagnosing bowel obstruction. Differences in sound characteristics between large bowel and small bowel obstruction may help determine the likely site of obstruction.

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Key words: Bowel sounds; Intestinal obstruction; Spectral analysis; Electronic stethoscope

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INTRODUCTION

Auscultation of bowel sounds is a traditional technique for evaluating patients with abdominal symptoms. It is simple, but generally empirical and too subjective. Bowel sounds show wide variations from person to person and even from the same person at different times. The interpretation of a person's bowel sounds by different clinicians may also vary[1,2]. There is a distinct lack of clinical research within the literature to support any discussion on the value of auscultation for bowel sounds[3]. Many practitioners pay little attention to this physical examination and some have even abandoned their stethoscope. The question has been raised as to whether auscultation for bowel sounds is of any clinical value.

Recently, recording of bowel sounds with objective evaluation has become possible with commercially available electronic stethoscopes; however, bowel sounds in intestinal obstruction have not been extensively studied since the introduction of these stethoscopes.

The aim of this study is to determine the value of objective assessment of bowel sounds provided by an electronic stethoscope in supporting a clinical diagnosis of intestinal obstruction. Correlation with radiological and operative findings and clinical outcome is made to identify characteristic bowel sounds that will improve the diagnostic accuracy for bowel obstruction.

MATERIALS AND METHODS

The electronic stethoscope used for this study was the 3M™ Littmann® Model 4100 with Version 2.0 sound analysis software (3M Health Care, St. Paul, MN 55144, United States). Three frequency modes are available for auscultation: bell (20-200 Hz), diaphragm (100-500 Hz), and extended range (20-1000 Hz). Bowel sounds are best heard in the diaphragm mode, which selectively filters in sounds of higher frequency range, thus omitting the low frequency sounds transmitted from the heart or lungs. Other features of the electronic stethoscope include the ability to amplify sounds several times compared with a conventional stethoscope, and digital signal processing over the entire acoustic range. A maximum of six distinct tracks of up to 8 s each can be recorded and stored in the stethoscope. The recorded sound tracks are then transmitted to a computer via an infrared link.

The Local Ethics Committee approved this study. The subjects were patients admitted to our institution with a diagnosis of possible intestinal obstruction during the period July 2009 to January 2011. Clinical data were prospectively collected. The presenting complaints and findings from physical examination were reviewed following admission to the ward. Verbal consent was obtained from the patients for recording of their bowel sounds. The patients were positioned lying supine on a bed. Six 8-s recordings were taken from each patient with the stethoscope positioned on the lower abdomen (two at the right iliac fossa, two at the suprapubic region, and two at the left iliac fossa). The reason for recording sounds from the three different areas of the lower abdomen was to position the stethoscope as far away from the heart and lungs as possible to reduce the interference by sounds arising from the heart and lungs. For the purpose of this study, no attempt was made to compare the sounds at different recording sites as a previous study showed that the frequency and intensity of bowel sounds were equal throughout all four quadrants[4]. The diagnosis of each of the patients was determined by clinical follow-up, clinical evolution, and by radiological and operative findings. The recorded sound tracks were analysed by the main investigator using the supplied software. The investigators were not blinded to the results of other tests such as radiological imaging or intraoperative findings.

When analyzing the sound tracks, the tracks were played back in the "diaphragm" mode. The overall quality of recording was determined by the level of ambient background noise that was actually present at the time of recording, and the presence of a constant "hissing" machinery noise (not present in the environment during recording), which could sometimes occur during playback of some of the tracks. This "ghost sound" phenomenon was noticeably more common when the entire track lacked actual bowel sounds and it was thought that the recording system compensates for the lack of sound by automatic over-amplification of the baseline machine noise. The waveform view displays the raw input signal as a continuous waveform line, where each wave is a pulse of sound. The maximum arbitrary amplitude (range 0% to 100%) of the bowel sounds was identified from each track (Figure 1).

Classification of bowel sounds

The sounds were described as "isolated" when they occurred in isolation and did not last for more than 0.5 s in duration and were more than 0.2 s apart. The spike of the isolated sound is shown as a single vertical line over a very short duration (Figure 1). The sounds were described as "clustered" when they occurred continuously for more than 0.5 s and recognised audibly as a run of individual "popping" effects (Figure 1), and "prolonged" when they occurred continuously for longer than 4 s[5]. The interval between sounds was determined by the period between the end of one sound and the start of the next sound. For the purpose of computation, when there were more than two distinct bowel sounds on a single track the shortest interval between two distinct bowel sounds was taken as the interval between sounds for that track. The spectrum view displays the frequency

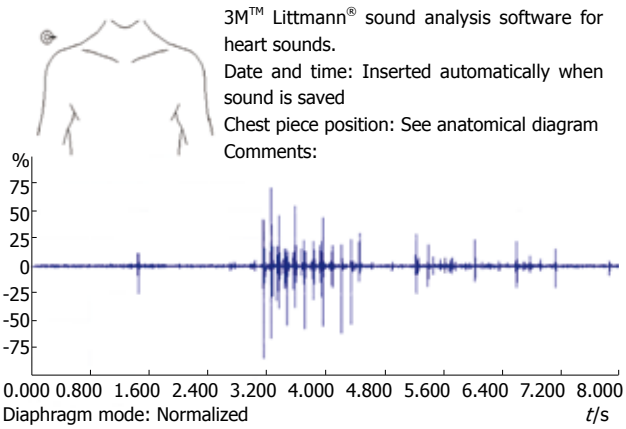


Figure 1 Example of bowel sound recording displayed in the "waveform" view. The y-axis indicates the relative sound amplitude and the x-axis indicates recording time elapsed in seconds. This recording was taken from a patient with a small bowel obstruction. Note the isolated sound on the left side followed by two clusters of sound in the middle and on right side of the strip.

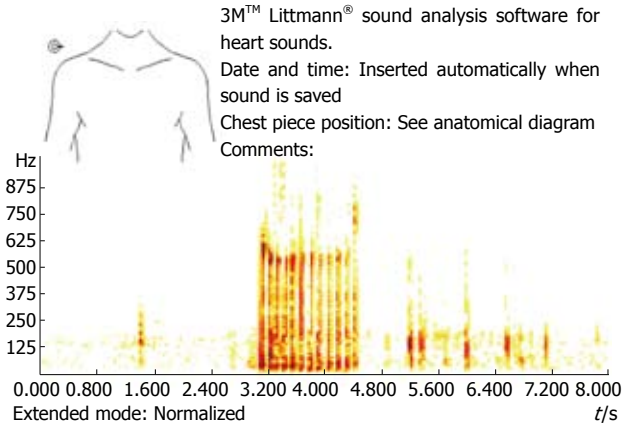


Figure 2 Example of bowel sound recording displayed in the "spectrum" view. This chart shows the sound frequency spectrum and the relative intensity of each sound frequency (darker colour indicates higher sound intensity at the frequency indicated along the y-axis) for the same recording used for Figure 1.

distribution as vertical bars, each bar represents a group of frequencies, and the more prominent frequencies have darker colour tones than the less prominent frequencies (Figure 2). The highest dominant frequency from each track was determined by first identifying the most dominant sound frequency across the spectrum at each point of time when bowel sounds were present. The highest dominant frequency over the entire duration of the track was then identified and used for analysis. The peak frequency was determined by the highest value dominant frequency among the six tracks for each individual patient.

X-rays and computed tomography (CT) imaging of the abdomen and pelvis were reviewed for evidence of intestinal distension. The calibre of the small bowel and large bowel at the widest points were determined using the measuring tool in the Amalga IMS 5.1 radiology image viewing system installed on our institution's computers. The point of transition was identified if bowel obstruction was present on CT images. Any operative intervention performed for the patients was also documented. The cause of obstruction, as identified from radiological imaging and/or during surgery, was recorded.

The following definitions were used to compare bowel sounds between patients with acute bowel obstruction *vs* subacute obstruction *vs* no obstruction[6-8]:

Acute bowel obstruction: Dilated bowel calibre (> 3 cm for small bowel, > 6 cm for large bowel) with symptoms of vomiting (for small bowel pathology) or no flatus/bowel motion for > 24 h (for large bowel pathology) and with CT/operative evidence of complete or high grade mechanical obstruction with a discrete transition point, collapsed distal segment, and little or no colonic gas present distal to the obstruction.

Subacute obstruction: Dilated bowel calibre (> 3 cm for small, > 6 cm for large bowel), with or without symptoms as per acute obstruction, but no CT/operative evidence of complete or high-grade mechanical obstruction (a poorly defined transition zone, incomplete collapse of distal bowel segment, and moderate colonic gas distal to the obstruction).

Acute (complete) or subacute (partial) mechanical obstruction of the bowel may be caused by herniation, adhesions, volvulus, intussusception, foreign body, phytobezoar, strictures, neoplasia, or wall oedema due to inflammatory conditions. Partial obstruction allows some liquid contents and gas to pass through the point of obstruction, whereas complete obstruction impedes passage of all bowel contents.

No obstruction: No clinical/CT/operative/endoscopic evidence of mechanical obstruction and other diagnosis was observed during the clinical course of the patient. Diagnoses such as constipation colic, faecal impaction, paralytic ileus, chronic megacolon, and pseudo-obstruction will be classified under this category.

Statistical analysis

Statistical analysis was performed using SPSS Version 12.0.1 (SPSS Inc. Chicago, Illinois, United States). Continuous non-parametric data were analysed using Mann-Whitney *U* test. A χ^2 test was used to correlate the type of bowel sounds with the presence of obstruction. Pearson correlation was used to correlate bowel calibre with sound duration, sound-to-sound interval, dominant frequency, and peak frequency. Continuous data was presented as median values with inter-quartile ranges.

RESULTS

Seventy-one patients were recruited and had their bowel sounds recorded during the period July 2009 to January 2011. These patients had been admitted to our institution with abdominal symptoms, physical signs, or radiological

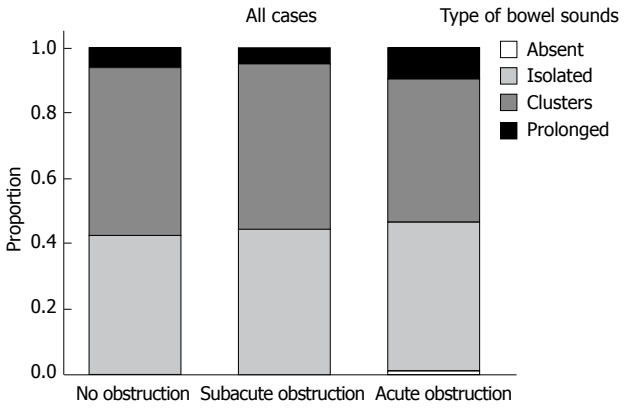


Figure 3 Distribution of different types of bowel sounds recorded for patients with no obstruction, subacute obstruction, and acute obstruction.

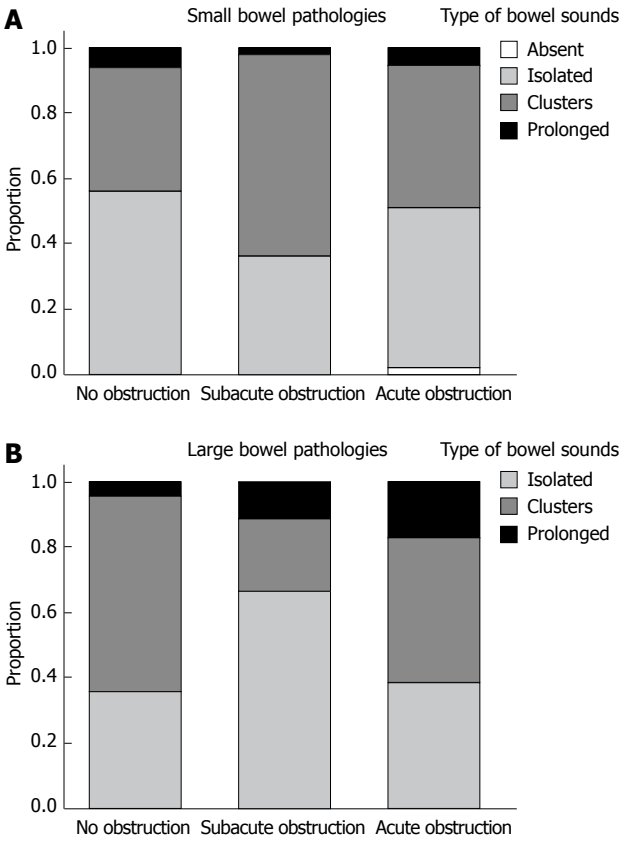


Figure 4 Distribution of different types of bowel sounds recorded for patients with small bowel pathologies (A) and large bowel pathologies (B).

findings suggestive of possible bowel obstruction.

There were 43 male and 28 female patients with age range from 22 to 93 years (mean 68 ± 16 years). Sixty-nine percent of patients presented with abdominal pain and 70.4% had a history of vomiting. Abdominal distension was present in 90.1% of the patients and abdominal tenderness was present in 33.8%. All patients had radiological imaging of the abdomen and pelvis. Plain film radiography was performed in all patients and CT was performed in 85.9% of patients.

Acute bowel obstruction was diagnosed in 40 patients (56.3%). Of these, 27 occurred in the small bowel and 13 occurred in the large bowel. Eleven patients (15.5%) had subacute bowel obstruction, of which eight occurred in the small bowel and three occurred in the large bowel. Twenty patients (28.2%) had no bowel obstruction. The diagnoses made for the non-obstructive conditions were ileus, pseudo-obstruction, gastritis, gastroenteritis, ileitis, colitis, constipation, cholecystitis, and bowel ischemia. Surgical intervention was performed in 25 patients (35.2%) during the same admission for the acute abdominal conditions.

Four hundred and twenty-six recordings were made from the 71 patients, of which 6 were of poor quality and were not used for analysis.

All types of bowel sounds were heard in all three groups of patients. Overall, isolated and clustered bowel sounds were heard most frequently in approximately equal proportions in all three groups of patients and prolonged sounds comprised 9.3% in the group with acute bowel obstruction (Figure 3).

The cases were further analysed separately in subgroups of patients with small bowel pathologies and patients with large bowel pathologies. In the subgroup of patients with small bowel pathologies, the incidence of prolonged sounds were 6%, 2% and 6% with no obstruction, subacute obstruction and acute obstruction, respectively, and were not significantly different (P = 0.208) (Figure 4A). In the subgroup of patients with large bowel pathologies, the incidence of prolonged sounds increased significantly from 4% with no obstruction to 11% with subacute obstruction, and 17% with acute obstruction (P = 0.025) (Figure 4B).

Among the groups of patients with no bowel obstruction, subacute obstruction, and acute obstruction, the distributions of dominant frequencies were similar, with the highest number of recordings with a dominant frequency in the 100 to 300 Hz range (Figure 5A). None of the recordings showed a dominant frequency of over 1000 Hz.

There was no significant difference between the sound characteristics in terms of sound duration (Figure 5B), sound-to-sound interval (Figure 5C), dominant frequency (Figure 5D), and peak frequency (Figure 5E) when comparisons were made among the groups of patients with acute bowel obstruction, subacute bowel obstruction, and no bowel obstruction. The results are summarised in Table 1.

In the patient subset with acute bowel obstruction (n = 40), 27 patients had a small bowel obstruction and 13 had a large bowel obstruction. The sound characteristics in acute small and large bowel obstruction were compared (Table 2). The sound duration was significantly longer (P = 0.021) and the dominant frequency significantly higher (P = 0.003) in the large bowel obstruction group. The median peak frequency was about 100 Hz higher in the large bowel obstruction group, but the difference did not reach statistical significance (P = 0.060).

Comparison was made between patients with acute large bowel obstruction and the three patients with

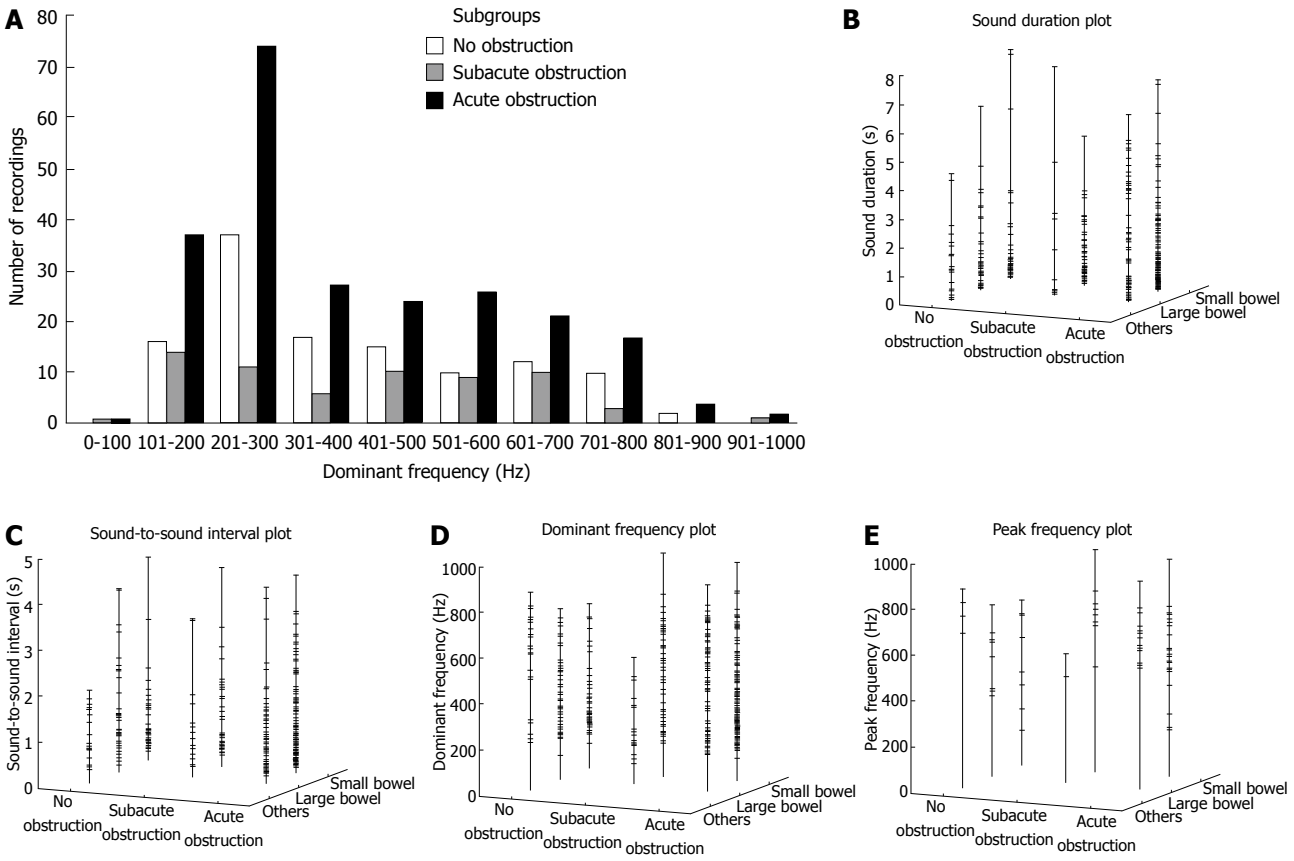


Figure 5 Distribution of the dominant frequencies of bowel sounds (A), sound duration (B), sound-to-sound interval (C), dominant frequency (D), and peak frequency (E) in patients with no obstruction, subacute obstruction, and acute obstruction, *etc*.

Table 1 Sound characteristics of acute bowel obstruction *vs* subacute bowel obstruction *vs* no obstruction

	No obstruction (n = 20)	Subacute obstruction (n = 11)	Acute obstruction (n = 40)	P value
Sound duration (s)	0.64 (0.20-1.57)	0.63 (0.23-1.67)	0.69 (0.19-2.10)	> 0.05
Sound-to-sound interval (s)	0.72 (0.46-1.27)	0.70 (0.47-1.67)	0.75 (0.41-1.41)	> 0.05
Dominant frequency (Hz)	325 (225-530)	405 (218-565)	315 (225-545)	> 0.05
Peak frequency (Hz)	595 (378-713)	655 (465-735)	585 (530-706)	> 0.05

Data are presented as the median (inter-quartile range).

Table 2 Sound characteristics of acute small bowel obstruction *vs* acute large bowel obstruction

	Small bowel obstruction (n = 27)	Large bowel obstruction (n = 13)	P value
Sound duration (s)	0.55 (0.17-1.67)	0.87 (0.27-3.60)	0.021
Sound-to-sound interval (s)	0.72 (0.41-1.40)	0.78 (0.44-1.42)	0.621
Dominant frequency (Hz)	288 (220-479)	440 (250-643)	0.003
Peak frequency (Hz)	560 (480-695)	660 (578-740)	0.060

Data are presented as the median (inter-quartile range).

pseudo-obstruction of the large bowel (Table 3). There was no significant difference in the sound characteristics between the two groups. Comparison between patients with a small bowel obstruction and a pseudo-obstruction (Table 4) showed significantly longer sound duration, longer sound-to-sound interval, and higher dominant frequency in patients with a pseudo-obstruction.

Analysis was performed on the 35 patients with a small bowel obstruction (27 acute obstruction and eight subacute obstruction included). Twenty-six patients (74%) with a small bowel obstruction were caused by (or presumed to be caused by) adhesions when there was a previous history of abdominal operation(s), CT (performed on 30 patients) did not find any mass lesion causing the obstruction, or the diagnosis confirmed during surgical exploration. Other causes of obstruction were small bowel volvulus (n = 3), bowel non-rotation (n = 1), femoral hernia (n = 1), parastomal hernia (n = 1), omental mass invasion (n = 1), and unknown (n = 2). The bowel sounds characteristics were compared between those who were treated conservatively (n = 25) *vs* those who were operated on (n = 10) (Table 5). The dominant frequency and peak frequency between non-operated and operated groups were not significantly different. The sound duration in the operated group was

Table 3 Sound characteristics of acute large bowel obstruction *vs* large bowel pseudo-obstruction

	Large bowel obstruction (n = 13)	Large bowel pseudo-obstruction (n = 3)	P value
Sound duration (s)	0.87 (0.27-3.60)	1.24 (0.53-2.64)	0.686
Sound-to-sound interval (s)	0.78 (0.44-1.42)	1.08 (0.68-2.23)	0.061
Dominant frequency (Hz)	440 (250-643)	488 (389-679)	0.174
Peak frequency (Hz)	660 (578-740)	630 (525-750)	0.638

Data are presented as the median (inter-quartile range).

Table 4 Sound characteristics of acute small bowel obstruction *vs* large bowel pseudo-obstruction

	Small bowel obstruction (n = 27)	Large bowel pseudo-obstruction (n = 3)	P value
Sound duration (s)	0.55 (0.17-1.67)	1.24 (0.53-2.64)	0.048
Sound-to-sound interval (s)	0.72 (0.41-1.40)	1.08 (0.68-2.23)	0.041
Dominant frequency (Hz)	288 (220-479)	488 (389-679)	0.001
Peak frequency (Hz)	560 (480-695)	630 (525-750)	0.446

Data are presented as the median (inter-quartile range).

longer than the non-operated group, but the difference did not reach statistical significance. The sound-to-sound interval was significantly longer in the operated group (P < 0.001).

Analysis of bowel sounds was made in relation to bowel dilatation in acutely obstructed bowel. There was no correlation between the small bowel calibre (4.8 ± 1.6 cm) and the sound duration (r = -0.046, P = 0.567), sound-to sound interval (r = -0.020, P = 0.817), dominant frequency (r = 0.025, P = 0.753), and peak frequency (r = -0.208, P = 0.298) for acute small bowel obstruction. Similarly, for acute large bowel obstruction, there was no correlation between the large bowel calibre (8.2 ± 1.7 cm) and sound duration (r = -0.103, P = 0.372), sound-to-sound interval (r = -0.077, P = 0.535), dominant frequency (r = -0.022, P = 0.847), and peak frequency (r = -0.028, P = 0.927).

DISCUSSION

Bowel sounds are generated by contractions of the alimentary tract, and mixing of gaseous and liquid contents[9]. The quality of bowel sounds varies according to the state of bowel activity[10,11]. Bowel sounds are complex, and each sound comprises a mixture of tones and is often a sequence of closely connected sounds. Common descriptions of bowel sounds include gurgling or rattling or rustling noise heard in a normal person, rumbling explosions heard with gastroenteritis, succussion splash heard in gastric outlet obstruction, diminished, i.e., infrequent, and soft sounds, and prolonged tinkling or high-pitched metallic sounds that may be heard in bowel obstruction. Very diminished or absent bowel sounds may be caused by bowel obstruction, intestinal ischemia, paralytic ileus, and peritonitis.

Table 5 Sound characteristics of non-operated and operated patients with small bowel obstruction

	Small bowel obstruction, non operated (n = 25)	Small bowel obstruction, operated (n = 10)	P value
Sound duration (s)	0.55 (0.21-1.45)	1.00 (0.25-2.26)	0.100
Sound-to-sound interval (s)	0.63 (0.39-1.13)	1.29 (0.61-1.82)	< 0.001
Dominant frequency (Hz)	360 (225-560)	265 (210-455)	0.084
Peak frequency (Hz)	625 (535-713)	525 (400-738)	0.432

Data are presented as the median (inter-quartile range).

The published literature on auscultation of bowel sounds has been rather scarce over the past century. Since Cannon described the rhythmic sounds produced by the stomach and intestines more than 100 years ago, very few papers have been published on this subject[12].

Advances in technology have allowed various systems to be developed for the objective analysis of bowel sounds. Spectral analysis of bowel sounds was first described by Horn *et al*[13] in 1966. The spectrogram that their apparatus produced was a complex record, which was difficult to understand, and the frequency range of their apparatus was too small. Work by Watson *et al*[14] in 1967 found that bowel sounds have a frequency range of at least 150 Hz to 5000 Hz, with peaks detected at frequencies of up to 2000 Hz. These very high frequencies were not seen in our study, where the peak dominant frequency of bowel sounds recorded never reached above 1000 Hz in any of our recordings.

Yoshino *et al*[15] in 1990 attempted a computer analysis of bowel sounds in intestinal obstruction. The number of subjects studied was small (n = 21). The peak frequency of patients with intestinal obstruction in the subgroup of patients that required surgery (612 ± 86 Hz, n = 5) was significantly higher compared with the subgroup that did not require surgery (273 ± 64 Hz, n = 3). The authors concluded that computer analysis of bowel sounds of mechanical obstruction could provide a very objective assessment of severity, and could help determine the treatment regimen (conservative or operative) of each patient. However, our data on patients with small bowel obstruction showed that the subgroup that subsequently underwent surgery (n = 10) did not have any significant difference in the dominant or peak frequencies when compared with the subgroup that did not undergo surgery (n = 25). Instead, there was a significantly longer sound-to-sound interval in the subgroup that underwent surgery compared with the subgroup that was treated conservatively. The explanation for this is difficult, but it may be that in situations

where the small bowel obstruction does not resolve and subsequently requires surgery, the affected bowel is more prone to ischemia and fatigue, becoming more distended and resulting in less frequent peristaltic activities and recordable sounds[10,16].

The study by Sugrue *et al*[17] in 1994 compared characteristics of bowel sounds in controls (n = 63) and patients with acute appendicitis (n = 25), acute cholecystitis (n = 15), and bowel obstruction (n = 21). This study compared sound number, duration, interval, and amplitude but not frequency of bowel sounds. It was found that in bowel obstruction, the sound duration, interval, and amplitude all significantly increased when compared to control subjects. However, in our current study, there was no significant difference in the sound duration and sound-to-sound interval between obstructed and non-obstructed bowel.

The current study is, so far, the largest series of patients with intestinal obstruction investigated for bowel sounds (n = 51). This study has shown objectively that bowel sound characteristics, in general, are not significantly different between patients with acute, subacute, or no intestinal obstruction using the commonly compared parameters, including sound duration, sound-to-sound interval, and dominant and peak frequencies. However, the obstructed large bowel has significantly longer sound duration (median 0.87 s *vs* 0.56 s) and higher dominant frequency (median 440 Hz *vs* 288 Hz) when compared to obstructed small bowel. The possible explanations for these differences are that movements within the colon usually involve less frequent peristalsis but larger volume shift with each peristalsis and, therefore, in the presence of an obstruction, a longer time elapses when fluid and gas are forced to pass through a tight stenosis. The higher sound frequency in large bowel obstruction can be explained by the fact that the majority of ileocecal valves are competent[18,19]. In the event of large bowel obstruction, the pressure rises within the "closed loop" segment of the colon proximal to the point of obstruction. Progressive distension of the colon increases the tension on the colonic wall, as well as thinning of the wall; hence, vibration is produced at a higher frequency. In small bowel obstruction, the pressure within the obstructed segment of bowel is usually limited by reflux of the small bowel contents back into the stomach (except in the presence of certain uncommon situations causing a closed loop obstruction), hence the common presentation of vomiting in small bowel obstruction.

In the small group of patients with large bowel pseudo-obstruction, the sound characteristics were similar to those with large bowel obstruction. This is not surprising, because in both conditions the large bowel can be grossly distended. The point of "obstruction" in patients with pseudo-obstruction is often caused by a high resting anal sphincter tone, which impedes the evacuation of flatus and faeces. This can be identified by digital rectal examination and the findings of a tight anus, which precludes easy entry of the examining finger. Upon entry of the finger, the rectum can be felt to be capacious and mainly gas and fluid filled.

The intrinsic difficulty with the study of bowel sounds is that it is never possible to hear or record exactly the same pattern of bowel sounds with exactly same amplitude, frequency, duration and interval repeatedly and consistently as compared to the consistent sounds produced from the heart, which has a set rhythm and sound characteristics over time. The bowel sounds heard from the same patient will also differ at different times when the patient is re-examined. Therefore intrasubject reproducibility of repeat recording was not evaluated in this study. The wide range of physiological variations means that the clinical significance of bowel sounds is limited. There is no clear evidence that high-pitched bowel sounds have clinical pertinence[20]. The recording of bowel sound yields only the sum of the motility of all areas of the alimentary track, so no statements are possible about the activity of any particular segment by comparison of sounds at different recording sites[21].

In this study, the recording of six 8-second tracts of bowel sounds performed over a few minutes may not be representative of the overall pattern over a longer period. The technical ability to perform continuous recording over a longer period is more likely to provide better representation of the whole picture, as well as improving intrasubject reproducibility. Recent biomedical engineering experimental studies have utilised bowel sounds to assess bowel motility and their techniques have shown good potential for monitoring and estimation of bowel motility[22-25]. Incorporation of these technologies into a portable electronic stethoscope might improve the recording, analysis, and interpretation of clinical data.

COMMENTS

Background

Auscultation of bowel sounds is a traditional technique for evaluating patients with abdominal symptoms. It is simple, but is generally empirical and too subjective. There is a distinct lack of clinical research to support the value of auscultation for bowel sounds.

Research frontiers

Recording of bowel sounds with objective evaluation has become possible with commercially available electronic stethoscopes. In this study, the authors aimed to determine the value of objective assessment of bowel sounds provided by an electronic stethoscope in supporting a clinical diagnosis of intestinal obstruction. Correlation with radiological and operative findings, and clinical outcome was made to identify characteristic bowel sounds that will improve the diagnostic accuracy for bowel obstruction.

Innovations and breakthroughs

This is the largest study examining bowel sounds for intestinal obstruction using a commercially available electronic stethoscope. Bowel sounds recorded for different conditions, including acute and subacute obstruction of the small bowel and large bowel, pseudo-obstruction of large bowel, and patients with no bowel obstruction were compared using measurable parameters, such as sound duration, sound-to-sound interval, dominant frequency, and peak frequency. The most important breakthroughs from this study are that large bowel obstruction has a significantly longer sound duration and higher dominant frequency than small bowel obstruction. Furthermore, bowel sounds in pseudo-obstruction of the large bowel mimic acute large bowel obstruction; therefore, auscultation is of less value in differentiating between these two conditions. Small bowel obstruction that resolved with conservative management had shorter sound-to-

sound interval than those that eventually needed surgery.

Applications

By understanding the differences in bowel sound qualities among various acute obstructive conditions of the bowel, practitioners can use their past experience and clinical judgement when auscultating and interpreting bowel sounds in an acute abdomen.

Terminology

Bowel sound duration, sound-to-sound interval, dominant frequency, and peak frequency are the main parameters used to analyse bowel sounds. The reasons for the observed differences among conditions, including acute and subacute obstruction, small and large bowel obstruction, were discussed.

Peer review

The authors assessed the value of objective analysis of bowel sounds in a unique group of patients who presented with a common, yet challenging, abdominal condition. The data presented in this study showed that auscultation of bowel sounds, seemingly so simple to perform, becomes very complex when subjected to a scientific study. Bowel sounds on their own may not be specific enough to provide a diagnosis of bowel obstruction. The combination of clinical and radiological assessments remains the standard for diagnosing bowel obstruction.

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BRIEF ARTICLE

Stopping or reducing dietary fiber intake reduces constipation and its associated symptoms

Kok-Sun Ho, Charmaine You Mei Tan, Muhd Ashik Mohd Daud, Francis Seow-Choen

Kok-Sun Ho, Charmaine You Mei Tan, Muhd Ashik Mohd Daud, Francis Seow-Choen, Seow-Choen Colorectal Surgery PLC, Singapore 238859, Singapore
Author contributions: Seow-Choen F designed the research; Tan CYM and Mohd Daud MA were responsible for data collection and analysis; Ho KS and Seow-Choen F wrote the paper.
Correspondence to: Francis Seow-Choen, MBBS, FRCSEd, FAMS, Seow-Choen Colorectal Surgery PLC, 290 Orchard Road, The Paragon No. 06-06, Singapore 238859, Singapore. seowchoen@colorectalcentre.com
Telephone: +65-67386887 Fax: +65-67383448
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Abstract

AIM: To investigate the effect of reducing dietary fiber on patients with idiopathic constipation.

METHODS: Sixty-three cases of idiopathic constipation presenting between May 2008 and May 2010 were enrolled into the study after colonoscopy excluded an organic cause of the constipation. Patients with previous colon surgery or a medical cause of their constipation were excluded. All patients were given an explanation on the role of fiber in the gastrointestinal tract. They were then asked to go on a no fiber diet for 2 wk. Thereafter, they were asked to reduce the amount of dietary fiber intake to a level that they found acceptable. Dietary fiber intake, symptoms of constipation, difficulty in evacuation of stools, anal bleeding, abdominal bloating or abdominal pain were recorded at 1 and 6 mo.

RESULTS: The median age of the patients (16 male, 47 female) was 47 years (range, 20-80 years). At 6 mo, 41 patients remained on a no fiber diet, 16 on a reduced fiber diet, and 6 resumed their high fiber diet for religious or personal reasons. Patients who stopped or reduced dietary fiber had significant improvement in their symptoms while those who continued on a high fiber diet had no change. Of those who stopped fiber completely, the bowel frequency increased from one motion in 3.75 d (± 1.59 d) to one motion in 1.0 d (± 0.0 d) ($P < 0.001$); those with reduced fiber intake had increased bowel frequency from a mean of one motion per 4.19 d (± 2.09 d) to one motion per 1.9 d (± 1.21 d) on a reduced fiber diet ($P < 0.001$); those who remained on a high fiber diet continued to have a mean of one motion per 6.83 d (± 1.03 d) before and after consultation. For no fiber, reduced fiber and high fiber groups, respectively, symptoms of bloating were present in 0%, 31.3% and 100% ($P < 0.001$) and straining to pass stools occurred in 0%, 43.8% and 100% ($P < 0.001$).

CONCLUSION: Idiopathic constipation and its associated symptoms can be effectively reduced by stopping or even lowering the intake of dietary fiber.

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Key words: Dietary fiber; Constipation; Chronic idiopathic constipation; Abdominal bloating

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INTRODUCTION

Lack of fiber in the diet was first postulated in 1971

as the cause of diseases such as diverticulosis, hemorrhoids and colorectal cancer[1]. Since then, partly due to widespread media publicity, it is now widely accepted that dietary fiber is a necessary component of a healthy diet and is required for normal bowel movement[2-5]. It is popularly used in the management of constipation by the public and by many doctors. Insoluble fiber is known to increase stool weight and decrease colonic transit time[6,7]. Fiber is said to aid in water retention in the colon and results in stools that are less dry and easier to evacuate. However, the reality is that stool moisture content remains at 70%-75% regardless of the amount of fiber and water consumed[7,8].

There is recent evidence that low fiber intake does not equate to constipation[9]. Patients with chronic constipation also have similar fiber intake to controls[10-13]. Patients with chronic constipation may also have worsening symptoms when dietary fiber intake is increased[14]. Another study found that lactulose was more effective in easing constipation when compared with fiber[15].

It has also been our experience that many patients with constipation are already consuming a large amount of fiber before they seek medical attention.

We therefore carried out a prospective longitudinal case study to investigate the effect of decreasing dietary fiber in patients with idiopathic constipation.

MATERIALS AND METHODS

Constipation was defined clinically in patients who presented either with symptoms of straining to expel bulky large stools or a bowel frequency of less than one motion per 3 d over a period of at least 3 mo. Patients who presented to the clinic with symptoms of constipation, abdominal distension, pain or bloating, difficulty in evacuation with or without symptoms of rectal bleeding were considered for the study. For the purpose of this study, we did not distinguish between slow colonic transit type or obstructed defecation type of constipation nor did we attempt to classify the patients according to irritable bowel syndrome subtypes. All the patients underwent colonoscopy to exclude colonic lesions. Patients who had colorectal cancer, previous colonic surgery, melanosis coli or thyroid disorders were excluded. Patients with anal conditions such as severe prolapsed hemorrhoids, chronic anal fissure or any other condition that required surgery were also excluded.

Sixty-three consecutive patients after normal colonoscopy were enrolled into the study from May 2008 to May 2010. Each patient was to act as their own control. The physiology of the gastrointestinal tract and the bulking effects of dietary fiber were explained to the patients[16,17]. Patients were then instructed to completely stop their intake of dietary fiber, including vegetables, cereals, fruits, wholemeal bread and brown rice for 2 wk. Those who were vegetarians were asked to eat white rice instead of unpolished rice, white bread instead of whole meal bread, and to take processed bean products for protein. They were to continue their normal quantities of carbohydrates and proteins. Sieved fruit juices and clear vegetable soups were allowed. Patients were instructed not to take any laxatives during these 2 wk. After 2 wk, patients were asked to continue on with as little fiber in their diet as they were comfortable with for the long term. Patients were followed up at 1 mo and 6 mo intervals and final results were analyzed at 6 mo.

Data collected included age, sex, general dietary fiber intake, symptoms of constipation, difficulty in evacuation of stools, anal bleeding, abdominal bloating or abdominal pain. Constipation was recorded as the interval in days between bowel movements. Difficulty in evacuation was a subjective measure and patients were asked to choose from one of 3 degrees (no straining, occasional or moderate straining and severe straining or straining most of the time).

Statistical analysis

All data was entered into a secured database, and accessed only by the authors. The paired samples *t* test was performed using SPSS for Windows (SPSS Inc., Chicago, United States), version 17.0 on an IBM personal computer. Results are expressed as mean ± SD.

RESULTS

There were 16 males (25.6%) and 47 (74.4%) females, median age 47 years (range, 20-80 years) included in the study. At the commencement of the study, all patients were already on a high fiber diet or taking fiber supplements. After 2 wk of a no fiber diet, patients were asked to continue on as little fiber in the diet as they were able to follow if this were to give them relief from their symptoms.

At 6 mo, 41 patients continued on a no fiber diet and 16 were on a reduced fiber diet. The remaining 6 patients continued on a high fiber diet for various reasons including being vegetarians or inability to stop consuming dietary fiber for religious or personal reasons.

The median age of patients who stayed on a no fiber diet was 46 years (range, 21-80 years), on a reduced fiber diet was 45 years (range, 20-65 years) and on a high fiber diet was 59 years (range, 28-75 years). There was no statistical significant difference in age between the 3 groups. There was also no statistical difference in sex between the 3 groups (Table 1).

At 6 mo follow-up, the interval between bowel movements decreased with the reduction in fiber intake ($P < 0.001$). Forty one patients who completely stopped fiber intake had their bowel frequency increased from one motion in 3.75 d (± 1.59 d) to one motion in 1.0 d (± 0.00 d) ($P < 0.001$). Of 16 patients who reduced their dietary fiber intake, 12 patients had daily bowel movement, 3 had one bowel movement every 2 to 3 d and one had a bowel movement every 4 to 6 d, giving one motion per 1.9 d (± 1.21 d) on a reduced fiber diet compared with 1 motion per 4.19 d (± 2.09 d) on a high fiber diet ($P < 0.001$). There was no change in the frequency of bowel movement for patients who continued with high dietary fiber intake, with one motion per 6.83 d (± 1.03 d) before and after consultation ($P = 1.00$).

Table 1 Age and sex of all patients, segregated by post-consultation dietary fiber intake

Variable	General (n = 63)	No fiber (n = 41)	P value	Reduced fiber (n = 16)	P value	High fiber (n = 6)	P value
Age (yr), mean (range)	47 (20-80)	46 (21-80)	0.864	45 (20-65)	0.459	59 (28-75)	0.052
Sex[1] n (%)	16 (25.4)	15 (36.6)	0.258	1 (6.25)	0.034	0 (0)	< 0.01

[1]Numbers of male in each group. Corresponding P values for the age and sex of each category to the overall mean are provided.

Table 2 Symptoms at presentation and at 6 mo following change in dietary fiber intake

Symptom	Symptoms at presentation (n = 63)	High dietary fiber (n = 6)	P value	Reduced dietary fiber (n = 16)	P value	No dietary fiber (n = 41)	P value
Anal bleeding	31	4	1	4	0.216	0	< 0.001
Constipation	63	6	1	12	0.041	0	< 0.001
Bloatedness	33	6	1	5	0.041	0	< 0.001
Strain in bowel opening	63	6	1	9	0.004	0	< 0.001
Abdominal pain	13	3	1	2	0.164	0	0.012

There was also a difference between the groups in the proportion of patients with associated symptoms. For symptoms of bloating, all of those on a high fiber diet continued to be symptomatic, while only 31.3% in the reduced fiber group and none of the no fiber group had symptoms (0%, $P < 0.001$) (Table 2).

With regards to straining, all those on a no fiber no longer had to strain to pass stools. Of those who reduced dietary fiber, 7 of 16 showed improvement while the symptoms remain unchanged in those who remained on a high fiber diet ($P < 0.001$ between groups).

Symptoms of abdominal pain only improved in patients who stopped fiber completely while those who continued on a high fiber diet or reduced fiber diet did not show any improvement (Table 2). In addition, those on a no dietary fiber diet no longer had symptoms of anal bleeding.

DISCUSSION

This study has confirmed that the previous strongly-held belief that the application of dietary fiber to help constipation is but a myth. Our study shows a very strong correlation between improving constipation and its associated symptoms after stopping dietary fiber intake. However whilst there was no significant difference between the mean age of the 3 groups with different post-consultation dietary fiber intake, older patients seemed less likely to stop dietary fiber, although this did not reach significance. We did not survey the actual reasons for resuming dietary fiber. The clinical impression during consultation however was that some of these patients were vegetarians, some felt uneasy not eating any fiber, whilst others could not completely discontinue fiber due to constant media and peer pressure to increase dietary fiber.

Constipation is often mistaken by the layman as the state of not passing stool, with the subsequent false notion that making more feces will allow easier defecation. In truth, constipation refers to the difficulty in evacuating a rectum packed with feces, and easier defecation cannot possibly be affected by increasing dietary fiber which increases bulky feces. In this paper, we looked at constipation both as the number of days before each motion as well as the ease of defecation.

It is well known that increasing dietary fiber increases fecal bulk and volume. Therefore in patients where there is already difficulty in expelling large fecal boluses through the anal sphincter, it is illogical to actually expect that bigger or more feces will ameliorate this problem. More and bulkier fecal matter can only aggravate the difficulty by making the stools even bigger and bulkier. Several reviews and a meta-analysis had already shown that dietary fiber does not improve constipation in patients with irritable bowel diseases[18-21].

The role of dietary fiber in constipation is analogous to cars in traffic congestion. The only way to alleviate slow traffic would be to decrease the number of cars and to evacuate the remaining cars quickly. Should we add more cars, the congestion would only be worsened. Similarly, in patients with idiopathic constipation and a colon packed with feces, reduction in dietary fiber would reduce fecal bulk and volume and make evacuation of the smaller and thinner feces easier. Adding dietary fiber would only add to the bulk and volume and thus make evacuation even more difficult.

Whilst it is often stated in physiology textbooks that bulking agents improve peristalsis, there is no proof of this in practice nor experimentally. Regardless of the food ingested, small intestinal and right mid colonic contents are fluid and all ingestible dietary fiber is suspended therein. Dietary fiber, therefore, cannot act as solid boluses for the initiation of peristalsis. In fact, dietary fiber had been shown to retard peristalsis and hold up gaseous expulsion in human experiments[22].

Dietary fiber is also associated with increased bloatedness and abdominal discomfort[22]. Insoluble fiber was reported to worsen the clinical outcome of abdominal pain and constipation[18-20]. In our recent study, patients who followed a diet with no or less dietary fiber intake showed a significant improvement, not just in their constipation,

but also in their bloatedness. Patients who completely stopped consuming dietary fiber no longer suffered from abdominal bloatedness and pain. These symptoms are caused by the fermentation of dietary fiber by colonic bacteria, which produces hydrogen, carbon dioxide and methane[23]. Gases that are trapped by peristaltic colon exert pressure on the walls, causing the abdominal pain experienced by patients. This was previously observed in a prior study on younger patients, when dietary fiber had been shown not to be effective in the management of children with recurrent abdominal pain or bloating[21].

Stools only become well-formed in the sigmoid colon and rectum and by this time, especially in constipated subjects, more stools result in more evacuation problems. It is not logical to increase both the volume and size of stool in patients with idiopathic constipation and indeed for anybody with difficulty in passing stools, e.g., due to anismus or anal spasm from anal stricture, fissure or pelvic outlet disorders. We have shown that decreasing the bulk and volume of feces immediately enables the easier evacuation of smaller and thinner stools through the anal sphincter mechanism. This eliminates the need to strain in passing stools, and prevents the tearing of the anal sphincter and bleeding due to large and bulky fecal loads. None of our patients experienced anal bleeding or straining following complete abstinence from dietary fiber.

The results of this study should lead us to reexamine popular beliefs in benefits of dietary fiber and more studies should be undertaken to confirm or repudiate these results.

In conclusion, contrary to popularly held beliefs, reducing or stopping dietary fiber intake improves constipation and its associated symptoms.

COMMENTS

Background

It is a widely accepted view that dietary fiber is essential for gut health and to promote bowel movements. However, most patients with chronic constipation seen by the authors were already taking high fiber diet with no improvement in their symptoms.

Research frontiers

The role of dietary fiber in patients with chronic constip is reevaluated.

Innovations and breakthroughs

The authors showed that reducing dietary fiber intake may actually improve symptoms of chronic constipation.

Applications

This could bring relief to millions of people suffering from chronic constipation in that reducing their dietary fiber intake may relieve their symptoms and suffering.

Peer review

This paper is a preliminary study looking at the effects of dietary fiber on the symptoms of constipation on a small cohort of cases. The discussion provided an overview of the evidence available in the literature on this topic and gave a fresh perspective on the benefits or harm of excessive dietary fiber. All in all, this is an interesting preliminary study that warrants publication and further research.

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BRIEF ARTICLE

Gastric mucin expression in *Helicobacter pylori*-related, nonsteroidal anti-inflammatory drug-related and idiopathic ulcers

Doron Boltin, Marisa Halpern, Zohar Levi, Alex Vilkin, Sara Morgenstern, Samuel B Ho, Yaron Niv

Doron Boltin, Zohar Levi, Alex Vilkin, Yaron Niv, Department of Gastroenterology, Rabin Medical Center, Tel Aviv University, Petah Tikva 49100, Israel
Marisa Halpern, Department of Pathology, Rabin Medical Center, Hasharon Hospital, Petah Tikva 49100, Israel
Sara Morgenstern, Department of Pathology, Rabin Medical Center, Beilinson Hospital, Petah Tikva 49100, Israel
Samuel B Ho, Department of Medicine, University of California, and VA San Diego Healthcare System, San Diego, CA 92161, United States
Author contributions: Boltin D acquired, analysed and interpreted the data and drafted the manuscript; Halpern M and Morgenstern S interpreted the histology; Levi Z performed the statistical analysis; Vilkin A and Ho SB critically reviewed the manuscript and provided important intellectual content; Niv Y supervised the study, assisted in the drafting of the manuscript, obtained funding and provided administrative, technical support.
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Correspondence to: Yaron Niv, Professor, Department of Gastroenterology, Rabin Medical Center, Tel Aviv University, 39 Jabotinski Street, Petah Tikva 49100, Israel. nivyaron80@gmail.com
Telephone: +972-3-9377237 Fax: +972-3-9210313
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Abstract

AIM: To determine the pattern of secreted mucin expression in *Helicobacter pylori* (*H. pylori*)-related, nonsteroidal anti-inflammatory drug (NSAID)-related and idiopathic gastric ulcers.

METHODS: We randomly selected 92 patients with *H. pylori*-associated (n = 30), NSAID-associated (n = 18), combined *H. pylori* and NSAID-associated gastric ulcers (n = 24), and patients with idiopathic gastric ulcers (n = 20). Immunohistochemistry for T-cell CD4/CD8, and for mucin 5AC (MUC5AC) and mucin 6 (MUC6), was performed on sections of the mucosa from the ulcer margin. Inflammation score was assessed according to the Sydney system.

RESULTS: MUC5AC was expressed on the surface epithelium (98.9%) and neck glands (98.9%) with minimal expression in the deep glands (6.5%). MUC6 was strongly expressed in the deep glands (97.8%), variable in the neck glands (19.6%) and absent in the surface epithelium (0%). The pattern of mucin expression in idiopathic ulcer margins was not different from the expression in ulcers associated with *H. pylori*, NSAIDs, or combined *H. pylori* and NSAIDs. CD4/CD8 ratio was higher in *H. pylori*-positive patients (P = 0.009). Idiopathic ulcers are associated with hospitalized patients and have higher bleeding and mortality rates.

CONCLUSION: Idiopathic ulcers have a unique clinical profile. Gastric mucin expression in idiopathic gastric ulcers is unchanged compared with *H. pylori* and/or NSAID-associated ulcers.

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Key words: Idiopathic ulcer; Mucin; Mucin 5AC; Mucin 6; *Helicobacter pylori*

Peer reviewer: Waliul Khan, MBBS, PhD, Mcmaster University, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection and non-steroidal anti-inflammatory drugs (NSAIDs) are the leading causes of peptic ulcer[1-5]. However, in up to 39% of cases neither of these risk factors is identified[6]. While it may be prudent to exclude rarer causes of gastric ulcer such as malignancy, Zollinger-Ellison syndrome or systemic mastocytosis, in many cases *H. pylori*/NSAID-negative ulcers are apparently idiopathic.

Mucins are high-molecular-weight glycoproteins, which are heavily decorated with O-linked oligo-saccharides and n-glycan chains, linked to a protein backbone. There are 21 mucin (*MUC*) genes known in the human genome. These genes encode 2 groups of mucins: secreted mucins and membrane-bound mucins. The main mucins expressed in the stomach are MUC 1 (membrane-bound) and MUC5AC and MUC6 (secreted mucins). It has been proposed that defects in gastric mucin quality or quantity play a role in the pathogenesis of *H. pylori*/NSAID-negative ulcers[7]. MUC5AC, forming the bulk of the adherent unstirred mucous layer, is secreted by surface foveolar cells, whereas MUC6 is secreted by neck and gland cells, and both are strongly expressed in normal gastric mucosa[8]. These two mucin proteins remain segregated within the mucous gel in a laminated linear arrangement[9]. NSAIDs disrupt the production of prostaglandin-E2 which mediates mucin secretion. *H. pylori* similarly decreases mucin synthesis *via* inhibition of galactosyltransferase[10], this despite mucin's inherent antibacterial properties[11].

The pattern of mucin secretion in idiopathic peptic ulcer disease has yet to be determined. The aim of the present study is to identify the clinical and endoscopic features, and gastric mucin secretion patterns, in peptic ulcer disease positive and negative for *H. pylori* infection and/or NSAID therapy.

MATERIALS AND METHODS

Patients

Approval was granted by the ethics committee of Rabin Medical Center. Non-consecutive patients who underwent routine or emergency upper endoscopy at the Department of Gastroenterology, Rabin Medical Center, Beilinson Hospital, and who were assigned an endoscopic diagnosis of gastric ulcer, between 2003 and 2009, were randomly identified using an established computerized endoscopy reporting system. Clinical parameters were recorded, including patient age and gender, major indication for upper endoscopy, concomitant diseases and the use of aspirin and NSAIDs in the previous 3 mo. Endoscopic parameters were recorded, including ulcer site, size and number. *H. pylori* status was determined *via* histological detection on biopsies taken from the ulcer margins, gastric body or antrum and/or the rapid urease test and/or ^{13}C-urea breath test performed within 3 mo. Exclusion criteria included cases where no biopsies were taken from the ulcer margin, and where biopsies revealed neoplasia. Patients with clinical or histological evidence of Zollinger-Ellison syndrome, gastrointestinal malignancy, eosinophilic gastroenteritis, systemic mastocytosis, and patients receiving bisphosphonates, potassium salts or iron, were excluded.

Tissue samples

Formalin-fixed, paraffin-embedded tissue samples from ulcer margins were obtained from the Pathology Department. Where additional biopsies of gastric body or antrum were taken at endoscopy, these too were obtained. Paraffin-embedded blocks were cut into 4 μm thick sections. Slides were deparaffinized in xylene and rehydrated using a graded ethanol series. Antigen was retrieved by boiling the slides in a microwave oven for 15 min in 0.01 mol/L citrate buffer (pH 6.0). Endogenous peroxidase was blocked with a 3% H_2O_2-methanol solution, and the slides were incubated in 10% normal goat serum for 30 min to prevent nonspecific staining. The tissue sections were then incubated overnight at 4 ℃ with primary antibody (MUC5AC or MUC6, 1:100; Santa Cruz, CA). The standard biotin-streptavidin-peroxidase method was then used, and the sections were lightly counterstained with hematoxylin. Histologically normal gastric biopsies were used as positive controls for MUC5AC and MUC6. The sections incubated with phosphate-buffered saline (0.01 mol/L, pH 7.4) instead of primary antibody were used as negative controls. Cytoplasm staining was assessed in at least 10 high-power fields by two blinded observers at 3 sites: the foveola, the mucous neck cells, and the glands. The range of cytoplasmic staining (0: 0%-5%; 1: 6%-30%; 2: 31%-60%; and 3: 61%-100%) and the intensity of staining (0: No staining; 1: Weak staining; 2: Intermediate staining; 3: Strong staining) were assessed, and averages of the grades were taken. The final staining score was defined as the product of scores for the range and intensity of cytoplasmic staining. Staining was defined as negative if the staining score was 0 or 1, intermediate for 2, 3 or 4, and high for 6 or 9[12]. All specimens were scored blindly.

Immunohistochemistry with monoclonal antibodies to T-cell CD4 and CD8 antigens was performed for 5 cases from each group using a technique previously described[13]. Tissue sections were cut 4 μm thick from routinely processed formalin-fixed and paraffin-embedded blocks. The slides were oven dried overnight at 60 ℃. The slides were then put inside the Ventana (Benchmark, United States). The Ventana was activated by loading the pre-programmed recipe file for the appropriate antibody. For CD4 we used polyclonal antibody (Spring, CA, United States), and for CD8 a monoclonal antibody, clone SP16 (DBS, CA, United States). Immunohistochemical staining was performed by the I-view DAB detection kit of Ventana. Dark brown staining was defined as positive, and no staining was defined as negative. Staining was scored as follows: 0 (no detectable staining); 1 (1%-10% positive cells); 2 (11%-50%); 3 (51%-80%); and 4 (more than 80%). In cells with positive staining, the staining was intense and uniform, so intensity was not factored

Table 1 Patient characteristics

	H. pylori+/NSAID- *n* = 30 (32.6%)	*H. pylori*-/NSAID+ *n* = 18 (19.6%)	*H. pylori*+/NSAID+ *n* = 24 (26.1%)	*H. pylori*-/NSAID- *n* = 20 (21.7%)	Total *n* = 92
Age, yr [mean (SD, range)]	58.6 (18.58, 18-88)	72.17 (10.44, 56-89)	69.46 (13.08, 24-83)	70.3 (12.64, 42-95)	66.6 (15.5, 18-95)
Gender (male) *n* (%)	10 (33)	9 (50)	13 (54.2)	12 (60)	44 (47.8)
Ethnicity *n* (%)					
Israeli (Jewish)	11 (36.7)	9 (50)	12 (50)	6 (30)	38 (41.3)
Israeli (Arab)	3 (10)	0 (0)	2 (8.3)	1 (5)	6 (6.5)
Western Europe/United States	7 (23.3)	0 (0)	1 (4.2)	2 (10)	10 (10.9)
Eastern Europe/FSU	2 (6.7)	6 (33.3)	6 (25)	6 (30)	20 (21.7)
Middle east/Africa	7 (23.3)	2 (11.1)	3 (12.5)	4 (20)	16 (17.4)
South America	0 (0)	1 (5.6)	0 (0)	1 (5)	2 (2.2)
Inpatient *n* (%)	11 (36.7)	12 (60)	10 (41.7)	16 (80)	49 (53.3)
Comorbid disease *n* (%)					
ASCVD	3 (10)	12 (66.7)	11 (45.8)	6 (30)	32 (34.8)
COPD	2 (6.7)	4 (22.2)	2 (8.3)	7 (35)	15 (16.3)
Diabetes	4 (13.3)	4 (22.2)	12 (50)	6 (30)	26 (28.3)
Current malignancy	1 (3.3)	2 (11.1)	2 (8.3)	2 (10)	7 (7.6)
Alcohol abuse	0 (0)	0 (0)	0 (0)	3 (15)	3 (3.3)
Other significant systemic disease	1 (3.3)[3]	1 (5.6)[4]	1 (4.2)[4]	5 (25)[5]	8 (8.7)
Total[1]	7 (23.3)	15 (83.3)	19 (79.2)	14 (70)	55 (59.8)
Primary indication for endoscopy *n* (%)					
Iron deficiency anemia	9 (30)	5 (27.8)	6 (25)	1 (5)	21 (22.8)
Epigastric pain/GERD	8 (26.7)	6 (33.3)	4 (16.5)	4 (20)	22 (23.9)
Upper GI bleeding	7 (23.3)	6 (33.3)	6 (25)	9 (45)	28 (30.4)
Fecal occult blood	2 (6.7)	0 (0)	1 (4.2)	1 (5)	4 (4.3)
Weight loss	2 (6.7)	1 (5.6)	5 (20.8)	0 (0)	8 (8.7)
Screening for gastric cancer	2 (6.7)	0 (0)	0 (0)	0 (0)	2 (2.2)
Esophageal varices	0 (0)	0 (0)	0 (0)	2 (10)	2 (2.2)
Vomiting	0 (0)	0 (0)	0 (0)	1 (5)	1 (1.1)
Other[2]	0 (0)	0 (0)	2 (8.3)	2 (10)	4 (4.3)
Hemoglobin, g/dL [mean (SD, range)]	11.0 (3.22, 3.4-16.7)	10.6 (2.77, 5.5-15.5)	11.0 (2.84, 5.7-14.7)	11.1 (2.70, 6.2-15.3)	10.9 (2.98, 3.4-16.7)
Died within 12 mo of endoscopy	3 (10)	2 (11.1)	2 (8.3)	5 (25)	12 (13.0)

[1]Number of patients with comorbidities as listed; [2]Fever unknown origin, gastric outlet obstruction, and surveillance following resection of gastric and esophageal carcinoma (1 case for each indication); [3]Inflammatory bowel disease; [4]Hemodialysis; [5]Inflammatory bowel disease, sepsis, cirrhosis (2 cases), tetraplegia following trauma. *H. pylori*: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drugs; ASCVD: Atherosclerotic cardiovascular disease; COPD: Chronic obstructive pulmonary disease; GERD: Gastro esophageal reflux disease; GI: Gastrointestinal. +: Positive; -: Negative.

into the scoring. The ratio of CD4/CD8 intraepithelial/mucosal lymphocytes was assessed for ten low-power microscopic fields. Inflammation score was measured according to the Sydney system, and compared between the groups[14]. Sydney score for inflammation is the sum of 5 criteria: *H. pylori* status, atrophy, intestinal metaplasia, lymphocytic infiltration and polymorphonuclear cell infiltration. Every criterion has 0 to 3 score (none exists, mild, moderate, severe) thus the range is 0 to 15.

Statistical analysis

Statistical analysis was performed using statistical package for the social sciences software 19.0 (SPSS, Inc.). Patient groups were compared using the Pearson χ^2 test, *F* test and Duncan test. *P* values were considered significant when $\leqslant 0.05$.

RESULTS

Ulcer biopsies from 92 patients were included in the final set analysis, including 30 *H. pylori*-associated ulcers, 18 NSAID-associated, 24 associated with combined *H. pylori*/NSAID and 20 idiopathic, neither associated with *H. pylori* nor NSAID use. Patient characteristics are summarized in Table 1.

Forty-four patients were male, with no significant differences between the 4 groups ($P = 0.24$). Mean age was 66.6 years. Patients with *H. pylori*-associated ulcers not receiving NSAIDs were significantly younger compared to the other 3 groups ($\alpha = 0.05$, Duncan test). There was no significant difference in the patients' origin between the groups. Although significant co-morbidities were observed in all groups, 80% (16/20) of patients with *H. pylori*/NSAID-negative ulcers were inpatients at the time of their endoscopy, compared to 45.8% (33/72) in other groups ($P = 0.007$). Furthermore, idiopathic ulcers were associated with decreased survival, where 25% (5/20) died within 12 mo, compared to 9.7% (7/72) in other groups (all cause mortality, $P = 0.04$).

Patients with *H. pylori*/NSAID-negative ulcers more often presented with upper gastrointestinal bleeding: 45% (9/20) compared with 26.4% (19/72) in the other groups ($P = 0.11$). Subacute and asymptomatic presentations (iron deficiency anemia, weight loss, fecal occult blood and screening) were less common in those with *H. pylori*/NSAID-negative ulcers (2/20, 10%, compared to 34/72,

Table 2 Endoscopic findings

	H. pylori+/NSAID- *n* = 30 (32.6%)	*H. pylori*-/NSAID+ *n* = 18 (19.6%)	*H. pylori*+/NSAID+ *n* = 24 (26.1%)	*H. pylori*-/NSAID- *n* = 20 (21.7%)	Total *n* = 92
Ulcer number (per procedure) *n* (%)					
1	21 (70)	10 (55.6)	13 (54.2)	14 (70)	58 (63.0)
2	4 (13.3)	3 (16.7)	5 (20.8)	3 (15)	15 (16.3)
⩾ 3	5 (16.7)	5 (27.8)	6 (25)	3 (15)	19 (20.7)
Ulcer size (*n*)[1]					
⩽ 5 mm	18	14	27	14	73
6-10 mm	7	8	10	11	36
11-20 mm	5	3	4	4	16
> 20 mm	4	0	0	2	6
Not specified	2	2	2	0	6
Ulcer location *n* (%)					
Antrum	19 (63.3)	10 (55.6)	18 (75)	16 (80)	63 (68.5)
Body	9 (30)	6 (33.3)	6 (25)	4 (20)	25 (27.2)
Cardia	2 (6.7)	2 (11.1)	0 (0)	0	4 (4.3)
Ulcer *H. pylori* positive *n* (%)	28 (93.3)[2]	0 (0)	20 (83.3)	0 (0)	48 (52.2)
Ulcer IM *n* (%)	5 (16.7)	4 (22.2)	3 (16.7)	4 (20)	16 (17.4)

[1]In 10 instances the endoscopist reported "ulcer number" as "multiple". Therefore the total number of ulcers is unknown and percentages cannot be calculated; [2]Cases where *H. pylori* was absent from ulcer margin, but had either positive rapid urease test, positive C13-urea breath test or positive histology in biopsy not taken from ulcer margin. NSAID: Non-steroidal anti-inflammatory drugs; *H. pylori*: *Helicobacter pylori*; IM: Intestinal metaplasia. +: Positive; -: Negative.

Table 3 Staining positivity for mucin 5AC and mucin 6 *n* (%)

		Mucin 5AC			Mucin 6		
		Surface cell positivity	Neck cell positivity	Gland cell positivity	Surface cell positivity	Neck cell positivity	Gland cell positivity
H. pylori+/NSAID-	a	29 (96.7)	29 (96.7)	2 (6.7)	0 (0)	4 (13.3)	29 (96.7)
(*n* = 30)	b	29 (96.6)	27 (90)	0 (0)	0 (0)	1 (3.3)	25 (83.3)
H. pylori-/NSAID+	a	18 (100)	18 (100)	2 (11.1)	0 (0)	4 (22.2)	17 (94.4)
(*n* = 18)	b	16 (88.9)	15 (83.3)	0 (0)	0 (0)	2 (11.1)	14 (77.8)
H. pylori+/NSAID+	a	24 (100)	24 (100)	2 (8.3)	0 (0)	5 (20.8)	24 (100)
(*n* = 24)	b	24 (100)	23 (95.8)	2 (8.3)	0 (0)	2 (8.3)	22 (91.7)
H. pylori-/NSAID-	a	20 (100)	20 (100)	0 (0)	0 (0)	5 (25)	20 (100)
(*n* = 20)	b	19 (95)	16 (80)	0 (0)	0 (0)	1 (5)	19 (95)
Total	a	91 (98.9)	91 (98.9)	6 (6.5)	0 (0)	18 (19.6)	90 (97.8)
(*n* = 92)	b	88 (95.7)	81 (88.0)	2 (2.2)	0 (0)	6 (6.5)	80 (97.0)

a: Positive stain defined as a stain extent and intensity score product ⩾ 2; b: Strongly positive stain, defined as a stain extent and intensity score product equalling 6 or 9. *H. pylori*: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drugs. +: Positive; -: Negative.

Table 4 Effect of *Helicobacter pylori* and non-steroidal anti-inflammatory drug status on mucin staining

	H. pylori+	*H. pylori*-	*P* value	NSAID+	NSAID-	*P* value
MUC5AC						
Surface cell positivity	53/54 (98.1)	38/38 (100)	0.39	42/42 (100)	49/50 (98)	0.36
Neck cell positivity	53/54 (98.1)	38/38 (100)	0.39	42/42 (100)	49/50 (98)	0.36
Gland cell positivity	4/54 (7.4)	2/38 (5.3)	0.68	4/42 (9.5)	2/50 (4)	0.29
MUC6						
Surface cell positivity	0/54 (0)	0/38 (0)	NA	0/42 (0)	0/50 (0)	NA
Neck cell positivity	9/54 (16.7)	9/38 (23.7)	0.40	9/42 (21.4)	9/50 (18)	0.68
Gland cell positivity	53/54 (98.1)	37/38 (97.4)	0.80	41/42 (97.6)	49/50 (98)	0.90

H. pylori: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drugs; NA: Not available. +: Positive; -: Negative.

47.2%, $P = 0.003$).

No difference between groups was observed regarding ulcer number or size. *H. pylori*-negative/NSAID-positive ulcers tended to be more often located in the gastric body: 6/18 (33.3%) compared with other groups 19/74 (25.7%) ($P = 0.51$). The presence of intestinal metaplasia did not differ between groups (Table 2).

Virtually all biopsies had positive (intermediate or high) staining for MUC5AC in the surface and neck cells (Figure 1). Staining for MUC5AC was minimal in gland cells (Tables 3 and 4). Although no significant difference between groups was observed there was a trend towards

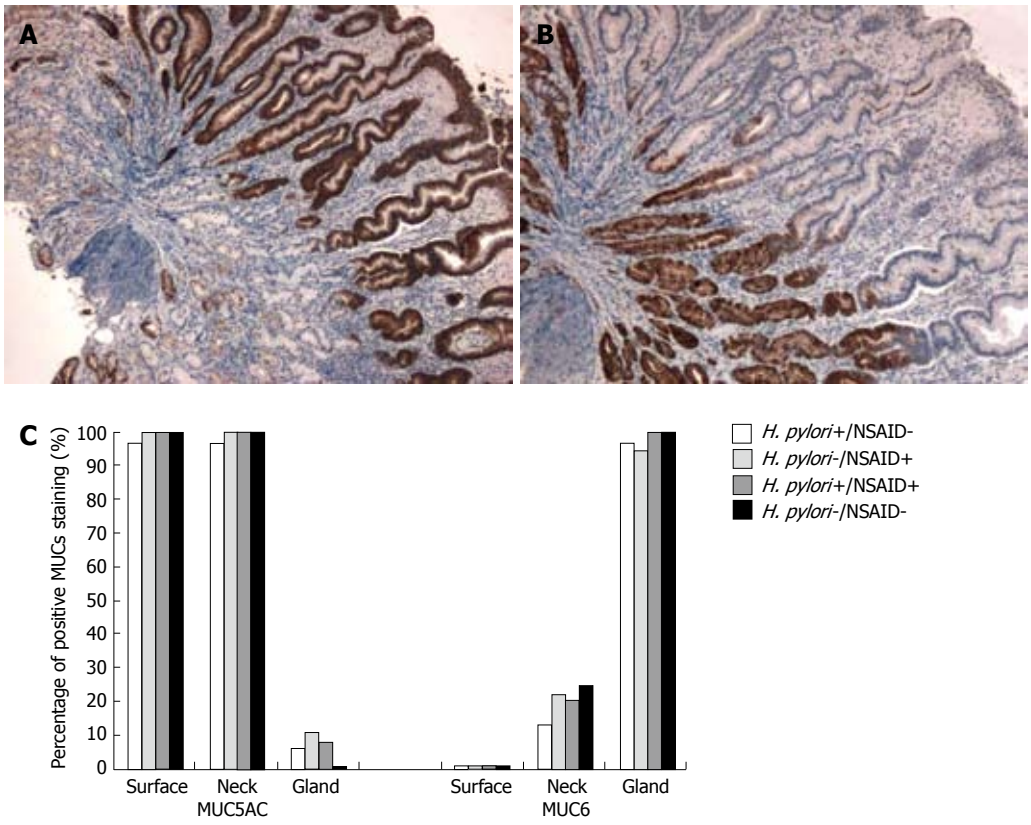


Figure 1 Mucin 5AC and mucin 6 expression in gastric ulcers. A: Immunohistochemical stain showing strong expression of mucin 5AC (MUC5AC) in surface and neck cells (× 10) of gastric mucosa from the ulcer edge of a patient with *Helicobacter pylori* (*H. pylori*)/non-steroidal anti-inflammatory drugs (NSAID)-negative gastric ulcer; B: Immunohistochemical stain showing strong expression of MUC6 in deep gland cells (× 10) of gastric mucosa from the ulcer edge of a patient with *H. pylori*/NSAID-negative gastric ulcer; C: Percentage of positive MUCs staining according to *H. pylori* and NSAID status. Positive stain defined as a stain extent and intensity score product ⩾ 2.

Table 5 Intraepithelial/mucosal T-cell populations (n = 20)

Group	CD4+/CD8+	CD8+/HPF	CD4+/HPF
H. pylori+/NSAID-	3.15	22.0 ± 4.4	69.6 ± 38.2[a]
H. pylori-/NSAID+	0.55	14.0 ± 9.4	8.4 ± 12.5
H. pylori+/NSAID+	2.99	26.6 ± 20.9	50.0 ± 25.2
H. pylori-/NSAID-	1.15	29.0 ± 11.4	38.0 ± 27.7

[a]P = 0.009 *vs H. pylori*-/NSAID+ group. *H. pylori*: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drugs; HPF: High-power field.

decreased expression of MUC5AC in the deep glands of idiopathic ulcers (P = 0.09). Including for analysis only cells with high mucin expression (extent and intensity score products of 6 or 9), and separate analyses of staining extent and intensity, did not significantly alter the results. All but two biopsies stained positively (intermediate or high) for MUC6 in the gland cells (Figure 1), and staining was uniformly negative in the surface cells. Staining for MUC6 was variable in the neck cells. Again, including only cells with high mucin expression (extent and intensity score products of 6 or 9), and separate analyses of staining extent and intensity, did not significantly alter the results.

T-cell CD4/CD8 ratio was significantly lower in the NSAID-positive groups when compared to the *H. pylori*-positive groups (P = 0.009, Table 5). As expected, the Sydney inflammation score was significantly lower in the *H. pylori*-negative/NSAID-negative group than in the *H. pylori*-positive/NSAID-negative group (P = 0.017, Table 6).

DISCUSSION

Gastric secreted mucin expression has not been previously studied in the setting of the idiopathic ulcer. In the present study, the distribution of immunohistochemical staining for MUC5AC and MUC6 in the margins of *H. pylori*/NSAID-negative ulcers did not significantly differ from staining patterns in the margins of ulcers associated with *H. pylori* and/or NSAIDS.

H. pylori interacts with gastric mucins in various manners, in order to facilitate its colonization. It has been established that *H. pylori* disrupts the assembly of the mucin molecule *via* inhibition of galactosyltransferase[10,12]. Furthermore, *H. pylori* reduces gastric mucous viscosity by elevating pH through urease secretion, thereby enhancing its motility within gastric mucous[15]. Kobayashi *et al*[16] demonstrated that BabA and SabA adhesins on *H. pylori* bind to Lewis B blood group antigens on MUC5AC, facilitating colonization.

On the other hand, gastric mucins have antimicrobial properties which are directed against *H. pylori*. Kawakubo *et al*[11] demonstrated that unique O-glycans in MUC6 inhibit bacterial biosynthesis of cholesteryl-α-*D*-glucopyranoside,

Table 6 Inflammation score according to the Sydney system (n = 20)

Group	*H. pylori*	Atrophy	Intestinal metaplasia	Lymphocytes	PMN	Score
H. pylori+/NSAID-	2.20 ± 0.44	0.60 ± 0.54	0.40 ± 0.54	1.80 ± 0.83	1.20 ± 1.30	6.20 ± 2.48[a]
H. pylori-/NSAID+	0.00 ± 0.00	0.40 ± 0.54	0.60 ± 1.34	1.40 ± 0.89	0.20 ± 0.44	2.60 ± 2.19
H. pylori+/NSAID+	1.80 ± 1.09	0.80 ± 0.83	0.40 ± 0.89	2.00 ± 0.70	1.40 ± 0.89	6.40 ± 3.13
H. pylori-/NSAID-	0.00 ± 0.00	0.20 ± 0.44	0.20 ± 0.44	1.40 ± 0.54	0.40 ± 0.54	2.20 ± 1.64

[a]P = 0.017 *vs H. pylori*-/NSAID- group. PMN: Polymorphonuclear cells; *H. pylori*: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drugs.

a major cell wall component. Lindén *et al*[17] suggest that mucins decorated with Le^b (the binding site for the *H. pylori* BabA adhesin) effectively bind *H. pylori*, thereby impairing its colonization of the mucosal surface. Similarly, in *Trichuris muris* infection MUC5AC is aberrantly expressed in the intestine and plays a key role in expulsion of the nematode[18]. Byrd *et al*[19] demonstrated that gastric biopsies from *H. pylori*-related gastritis patients frequently expressed the aberrant location of MUC6 staining in the surface foveolar cells. In the present study, cytoplasmic mucin staining of ulcers associated with *H. pylori* was unchanged. One reason for this discrepancy is the fact that the present study only included patients with gastric ulcers, and sampled gastric mucosa immediately adjacent to the edge of a gastric ulcer. These patients represent a distinct subgroup of patients with *H. pylori* infection, and our findings imply that alterations in secreted gastric mucins do not play a role in the pathogenesis of gastric ulcer in patients with *H. pylori* infection. This is supported by Marques *et al*[20] who found that although topographic expression of MUC5AC and MUC6 mucins was altered in *H. pylori*-related gastritis, the expression of these mucins was unchanged in mucosa adjacent to patients with *H. pylori*-related gastric ulcer.

An interesting finding in our study was that many patients with *H. pylori*/NSAID-negative ulcers had multiple comorbidities, were more often inpatients at the time of endoscopy, had fewer subacute presentations, and had a poorer survival. This concurs with Chan *et al*[21] who noted that three-quarters of patients with acutely bleeding *H. pylori*/NSAID-negative ulcers have significant comorbidity including major organ failure and malignancy. A large prospective study found that concomitant diseases and the absence of epigastric pain are independent risk factors for *H. pylori*/NSAID-negative ulcers in the duodenum[22]. A higher number of comorbidities were associated with increased ulcer size and depth, and more bleeding complications. Furthermore, our data are consistent with previous findings that idiopathic ulcer is an independent risk factor associated with long-term mortality[23,24].

In this study, *H. pylori*/NSAID-negative gastric ulcers were associated with underlying systemic disease, which could be severe. This association has been reported previously in idiopathic ulcers[24], and suggests the possible role of ischemic or non-specific inflammatory factors in their pathogenesis[25]. Despite the heterogeneity of possible pathogenic factors of these idiopathic *H. pylori*/NSAID-negative cases, our results indicate virtually no difference in qualitative MUC5AC and MUC6 staining in these ulcers compared with definite *H. pylori*-positive, NSAID-positive, and combined *H.pylori*/NSAID-positive gastric ulcers .

H. pylori-positive ulcer is associated with a high inflammation rate, thus groups 2 and 4 had significantly lower Sydney inflammation score than groups 1 and 3 (P = 0.017). In addition, we found a low ratio of T-cell CD4/CD8 in the groups negative for *H. pylori*; but when NSAID use was also negative the result did not reach significance. Similar findings were described by Strömberg *et al*[26]. In peptic ulcer patients positive for *H. pylori*, the number of intraepithelial T-cell CD4+ was higher than in patients with *H. pylori* infection but without ulcer or in healthy controls negative for *H. pylori*. Thus, *H. pylori* infection recruits CD4+ lymphocytes.

A limitation of our study is the retrospective nature of the data collection, which precluded elimination of false negative tests for *H. pylori* (probably due to proton pump inhibitor, bismuth or antibiotics), and cases of surreptitious or unreported NSAID use, which would result in misclassification of ulcers as *H. pylori*/NSAID-negative. This could only be overcome using a prospective study design, by performing multiple tests for *H. pylori* and assaying serum salicylate and plasma thromboxane, respectively.

In conclusion, patterns of mucin secretion in *H. pylori*/NSAID-negative ulcers need to be further studied in well-designed, prospective studies which minimize cross-contamination of groups. Idiopathic peptic ulcers are an increasingly encountered entity, with unique clinical and endoscopic features. Future efforts should focus on identifying genetic and epigenetic factors which regulate mucin secretion in this setting, as well as characterizing a potential role of the membrane-bound mucins and other mucosal protective factors.

COMMENTS

Background

In health, a mucin layer protects the stomach from the harmful effects of gastric acid and ulceration. The main causes of peptic ulcer disease are *Helicobacter pylori* (*H. pylori*) infection and aspirin or nonsteroidal anti-inflammatory drug (NSAID) therapy. However, up to 39% of peptic ulcer disease is idiopathic. Idiopathic ulcers are often associated with poorer outcomes.

Research frontiers

Alterations in secreted gastric mucins have been described in *H. pylori* infection and NSAID treatment, and may have a role in ulcer pathogenesis. This is the first study to look at gastric mucin secretion in idiopathic ulcers.

Innovations and breakthroughs

Mucin 5AC (MUC5AC) and MUC6 are equally expressed by *H. pylori*-induced,

NSAID-induced and idiopathic ulcers. The number of CD4 and CD8 lymphocytes is highest in *H. pylori*-induced ulcers reflecting a greater inflammatory component. Inflammation score is lowest in NSAID-associated ulcers.

Applications

Idiopathic peptic ulcer disease is more aggressive than peptic ulcer disease induced by *H. pylori* or NSAIDs. Elucidating the pathogenesis of idiopathic ulcers will lead the way to finding appropriate therapies.

Terminology

Mucin is the major component of gastric mucous and is synthesized and secreted by specialized cells. The two primary gastric mucin molecules are coined MUC5AC and MUC6; *H. pylori* is a ubiquitous bacterium which may cause gastric ulcer; NSAIDs include a range of over-the-counter medications commonly used for analgesia and rheumatic disease, and similarly cause gastric ulceration. Idiopathic ulcers are ones with no identifiable cause.

Peer review

The manuscript is interesting and has potential to enhance understanding on MUC5AC and MUC6 expression in gastric mucosa in *H. pylori*-, NSAID- and idiopathic-ulcers.

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BRIEF ARTICLE

What MELD score mandates use of entecavir for ACLF-HBV HBeAg-negative patients?

Ying Yan, Li Mai, Yu-Bao Zheng, Shao-Quan Zhang, Wen-Xiong Xu, Zhi-Liang Gao, Wei-Min Ke

Ying Yan, Li Mai, Yu-Bao Zheng, Shao-Quan Zhang, Wen-Xiong Xu, Zhi-Liang Gao, Wei-Min Ke, Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, Guangdong Province, China
Author contributions: Yan Y and Mai L contributed equally to this work; Yan Y, Mai L and Ke WM designed the research; Yan Y, Mai L, Zheng YB, Zhang SQ, Xu WX and Gao ZL performed the research; Yan Y, Mai L, Zheng YB, Zhang SQ and Xu WX collected data; Yan Y and Mai L analyzed the data; Yan Y, Mai L and Ke WM wrote the paper.

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Correspondence to: Wei-Min Ke, Professor, Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, Guangdong Province, China. kwm1999@163.com
Telephone: +86-20-85253172 Fax: +86-20-85253172
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Abstract

AIM: To investigate optimal timing for therapeutic efficacy of entecavir for acute-on-chronic hepatitis B liver failure (ACLF-HBV) in hepatitis B e antigen (HBeAg)-negative patients.

METHODS: A total of 109 inpatients with ACLF-HBV were recruited from the Department of Infectious Diseases of the Third Affiliated Hospital, Sun Yat-sen University from October 2007 to October 2010. Entecavir 0.5 mg/d was added to each patient's comprehensive therapeutic regimen. Patients were divided into three groups according to model for end-stage liver disease (MELD) score: high ($\geqslant$ 30, 20 males and 4 females, mean age 47.8 ± 13.5 years); intermediate (22-30, 49 males and 5 females, 45.9 ± 12.4 years); and low ($\leqslant$ 22, 28 males and 3 females, 43.4 ± 9.4 years). Statistical analysis were performed using SPSS 11.0 software. Data with normal distribution were expressed as mean ± SD and comparisons were made with Student's *t* tests. A value of $P < 0.05$ was considered statistically significant. Viral loads were related exponentially and logarithmic data were used for analysis.

RESULTS: For 24 patients with MELD score $\geqslant$ 30, treatment lasted 17.2 ± 16.5 d. Scores before and after treatment were significantly different (35.97 ± 4.87 and 40.48 ± 8.17, respectively, $t = -2.762$, $P = 0.011$); HBV DNA load was reduced (4.882 ± 1.847 copies $\log_{10}$/mL to 3.685 ± 1.436 copies $\log_{10}$/mL); and mortality rate was 95.83% (23/24). Of 54 patients with scores of 22-30, treatment lasted for 54.0 ± 43.2 d; scores before and after treatment were 25.87 ± 2.33 and 25.82 ± 13.92, respectively ($t = -0.030$, $P = 0.976$); HBV DNA load decreased from 6.308 ± 1.607 to 3.473 ± 2.097 copies $\log_{10}$/mL; and mortality was 51.85% (28/54). Of 31 patients with scores $\leqslant$ 22, treatment lasted for 66.1 ± 41.9 d; scores before and after treatment were 18.88 ± 2.44 and 12.39 ± 7.80, respectively, ($t = 4.860$, $P = 0.000$); HBV DNA load decreased from 5.841 ± 1.734 to 2.657 ± 1.154 copies $\log_{10}$/mL; and mortality was 3.23% (1/31).

CONCLUSION: For HBeAg-negative patients with ACLF-HBV, when entecavir was added to comprehensive therapy, a MELD score $\geqslant$ 30 predicted very poor prognosis due to fatal liver failure.

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Key words: Acute-on-chronic hepatitis B liver failure; Hepatitis B e antigen negativity; Entecavir; Model for end-stage liver disease; Mortality

Peer reviewer: Tatsuya Ide, MD, PhD, Assistant Professor, Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan

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INTRODUCTION

Currently, there are only four commercially available nucleoside analogs for the treatment of hepatitis B virus (HBV). These include lamivudine, adefovir dipivoxil, entecavir and telbivudine; all efficient antiviral agents that can reduce the HBV DNA load to the lower limit of detection in 72%, 51%, 90% and 88% of hepatitis B e antigen (HBeAg)-negative chronic hepatitis B (CHB) patients, respectively, after 1 year of treatment[1-5]. However, these drugs seem to be ineffective for some patients with acute-on-chronic hepatitis B liver failure (ACLF-HBV), for whom even comprehensive therapy in combination with antiviral treatment cannot reduce a high mortality rate[6,7]. When compared with HBeAg-positive CHB patients, the proportion of HBeAg-negative CHB patients has been increasing during recent years, and the age of these patients is relatively advanced. Although the HBV-DNA load is relatively low, this form of hepatitis is active, prone to fluctuation, and positively correlated with the degree of inflammation and necrosis of the liver[8,9]. Furthermore, cirrhosis and hepatocellular carcinoma are more prevalent among these patients. In a previously reported study, 71.74% of patients with ACLF-HBV were negative for HBeAg[6]. Here, we evaluated the efficacy of entecavir, a potent antiviral nucleoside analog, in ACLF-HBV patients having different model for end-stage liver disease (MELD) scores, and suggest a new method for guiding the therapeutic decision-making process.

MATERIALS AND METHODS

Patients

A total of 109 inpatients with ACLF-HBV were recruited from the Department of Infectious Diseases of the Third Affiliated Hospital, Sun Yat-sen University from October 2007 to October 2010. Diagnosis was based on the Guidelines for Diagnosis of Liver Failure (2006)[10] and included the presence of two or more of the following: prothrombin time/international normalized ratio (PT-INR) $\geqslant$ 1.5; serum total bilirubin > 10 times the upper limit of normal; ascites; hepatic encephalopathy; decreased liver size; or the hepatorenal syndrome. The standard serum creatinine (Cr) was defined as the normal median (31.8-93.7 μmol/L), and MELD scores were $\geqslant$ 16.1. All patients were hepatitis B surface antigen (HBsAg)-positive, HBeAg-negative, hepatitis B e antibody (HBeAb)-positive and hepatitis B core antibody (HBcAb)-positive. There were 97 male (89.0%) and 12 female (11.0%) patients with a mean age of 45.61 ± 11.90 years (range: 25-73 years). Patients were divided into three groups according to MELD score: high ($\geqslant$ 30, 20 male and 4 female, mean age 47.8 ± 13.5 years); intermediate (22-30, 49 male and 5 female, 45.9 ± 12.4 years); and low ($\leqslant$ 22, 28 male and 3 female, 43.4 ± 9.4 years). There were no marked differences in age and sex among the three groups. Patients co-infected with hepatitis A, C or E virus or human immunodeficiency virus were excluded. None had been treated with regular pegylated interferon, nucleoside analogs, or thymosin. Entecavir 0.5 mg/d was added to each patient's comprehensive therapeutic regimen as the sole antiviral agent.

Detections

HBV DNA levels were determined by real-time polymerase chain reactions with commercial diagnostic kits (Da-an GeneCo., Guangzhou, China). The lower detection limit was 500 copies/mL. HBsAg, HBsAb, HBeAg, HBeAb and HBcAb were detected with an automatic rapid immunoassay system (AxSYM; Abbott, United States). Total bilirubin and Cr and were measured using an automatic biochemical analyzer (AU 640; Olympus, Japan); normal ranges were 4-17.1 μmol/L and 31.8-93.7 μmol/L, respectively. PT-INR = (prothrombin time/reference prothrombin time) ISI. Prothrombin time was measured using detection reagent STA-Neoplastine(r) CI PLUS with an automatic coagulometer (STA-R) (Diagnostica Stago, France). Sample collection, transportation, preservation and processing were performed according to the manufacturer's instructions.

Calculations of MELD scores

MELD score = 3.8 × $\log_e$ [serum bilirubin (μmol/L) × 0.058] + 11.2 × $\log_e$ (PT-INR) + 9.6 × $\log_e$ [serum Cr (μmol/L) × 0.011] + 6.4 × (0 or 1) (cholestatic or alcoholic cirrhosis: 0; other liver diseases: 1)[11].

Definitions of therapeutic efficacy

Survival: patients discharged with no evidence of liver failure; death: progression of disease leading to death in spite of comprehensive therapy and entecavir treatment.

Statistical analysis

Statistical analysis were performed using SPSS 11.0 software. Data with normal distribution were expressed as mean ± SD and comparisons were made with Student's *t* tests. A value of $P < 0.05$ was considered statistically significant. Viral loads were related exponentially and logarithmic data were used for analysis.

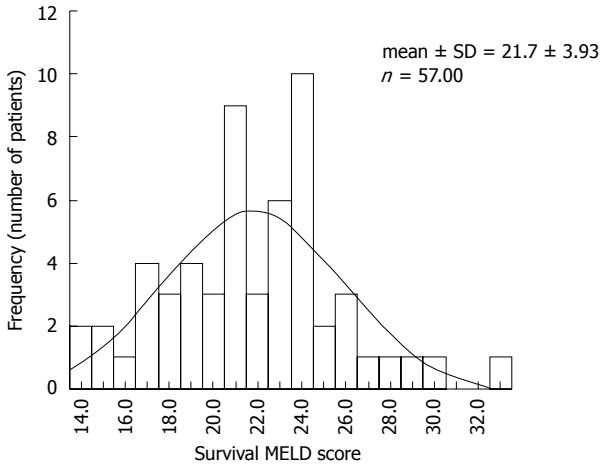
RESULTS

The mortality rate was > 95% in patients with MELD score > 30, and > 95% of patients survived with MELD

A

Statistics

Survival		
n	Valid	57
	Missing	14
mean		21.67867
median		21.54514
Percentiles	5	14.60103
	25	18.74029
	50	21.54514
	75	23.75528
	95	28.63190
	96	29.38532
	97	30.69348



B

Statistics

Death		
n	Valid	52
	Missing	19
mean		30.9545
median		29.1479
Percentiles	3	21.9790
	4	22.2608
	5	22.8505
	25	27.6117
	50	29.1479
	75	34.6216
	95	43.9692

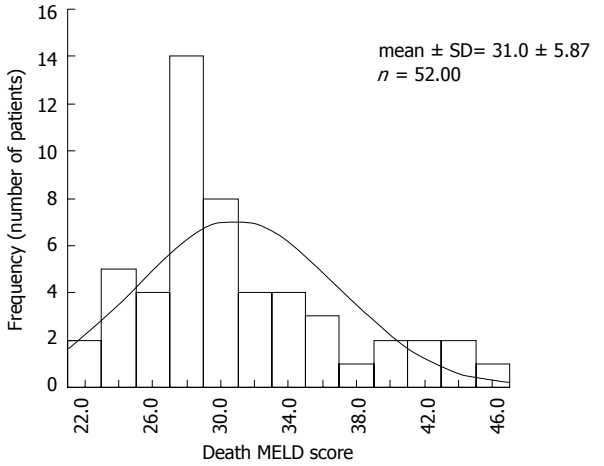


Figure 1 Frequency distribution of model for end-stage liver disease scores of patients receiving entecavir who survived (A) or succumbed (B). Death occurred in 95% of patients with MELD score > 28.63, 96% with score > 29.39 and 97% with score > 30.69; and in 5% with score < 22.85, 4% with score < 22.26, and 3% with score < 21.97. The mortality rate was > 95% in patients with MELD score > 30, and > 95% patients survived with MELD score < 22. MELD: Model for end-stage liver disease.

score < 22. Frequency distribution of MELD scores of patients receiving entecavir who survived or succumbed is described in Figure 1.

Of the 24 patients with high MELD scores (≥ 30), 23 (95.83%) died. Antiviral treatment was administered for 17.2 ± 16.5 d. Comparing measurements taken before antiviral treatment and before death, there were no marked differences in HBV DNA load, total bilirubin, INR and prothrombin activity (PTA), but Cr and MELD scores increased significantly (P = 0.003 and 0.011, respectively) (Table 1).

Of the 54 patients with intermediate MELD scores, 28 (51.85%) died and 26 survived. Antiviral treatment was administered for 54.0 ± 43.2 d. The MELD scores before and after antiviral treatment were not significantly different (25.87 ± 2.33 and 25.82 ± 13.92, respectively; t = -0.030, P = 0.976), but the HBV DNA load decreased significantly, by 2.918 ± 1.811 copies $\log_{10}$/mL (P = 0.000). Liver function results and outcomes are presented in Table 1.

There were 31 patients with low MELD scores; one (3.23%) died and 30 survived. Antiviral treatment was administered for 66.1 ± 41.9 d. Total bilirubin decreased and PTA increased significantly during antiviral treatment (P < 0.05), and decreases in MELD score (18.88 ± 2.44 to 12.39 ± 7.80; t = 4.860, P = 0.000) and HBV DNA levels (by 3.185 ± 1.740 copies log10/mL, P = 0.000) were also significant (Table 1).

DISCUSSION

Providing comprehensive treatment is the main therapeutic strategy for patients with ACLF; when possible other treatment options include use of bioartificial liver support systems and liver transplantation. Comprehensive treatment can only prolong survival for the fraction of patients having a potential for liver regeneration. When antiviral treatment is added, prolonged survival times can be achieved at low cost. It remains controversial whether comprehensive therapy with bioartificial liver support systems is translated into a survival benefit. Liver transplantation is usually limited by high cost, insufficient donors, and unclear effects on long-term survival. In recent years, treatment of liver failure with stem cell transplantation has achieved favorable outcomes in preclinical studies, but this strategy has not been approved and its

Table 1 Models for end-stage liver disease score group: Liver function, hepatitis B virus DNA load, length of treatment and mortality rate

n = 24	Total bilirubin (μmol/L)	INR	Prothrombin activity (%)	Blood creatinine (μmol/L)	MELD score	HBV DNA loads (copies log10/mL)	Treatment course (d)	Mortality (%)
High model (*n* = 24)								
Before treatment	563.8 ± 163.0	4.14 ± 1.05	21.2 ± 5.8	124.0 ± 113.1	35.97 ± 4.87	4.882 ± 1.847	17.2 ± 16.5	95.83 (23/24)
Before death	615.3 ± 216.4	4.67 ± 1.71	20.0 ± 8.1	179.1 ± 129.1[b]	40.48 ± 8.17	3.685 ± 1.436		
t value	-1.742	-1.699	0.738	-3.340	-2.762	2.590		
P value	0.095	0.103	0.468	0.003	0.011	0.027		
Intermediate model (*n* = 54)								
Before treatment	444.5 ± 160.3	2.56 ± 0.71	35.6 ± 12.1	70.1 ± 15.8	25.87 ± 2.33	6.308 ± 1.607	54.0 ± 43.2	51.85 (28/54)
After treatment	409.5 ± 357.7	3.23 ± 2.26	38.4 ± 24.1	93.4 ± 75.8	25.82 ± 13.92	3.473 ± 2.097[b]		
t value	0.667	-2.637	-1.132	-2.223	-0.030	8.826		
P value	0.508	0.011	0.263	0.031	0.976	0.000		
Low model (*n* = 31)								
Before treatment	351.9 ± 114.8	1.59 ± 0.37	56.8 ± 14.1	62.5 ± 11.1	18.88 ± 2.44	5.741 ± 1.734	66.1 ± 41.9	3.23 (1/31)
Recovery stage	102.8 ± 144.4	1.55 ± 0.64	63.5 ± 18.2	67.4 ± 52.6	12.39 ± 7.80	2.657 ± 1.154		
t value	7.065	0.411	-2.468	-0.526	4.860	9.332		
P value	0.000	0.684	0.020	0.603	0.000	0.000		

Before death meaning: laboratory findings before articulo mortis. [b]$P < 0.01$ *vs* before treatment. MELD: Model for end-stage liver disease; HBV: Hepatitis B virus; INR: International normalized ratio.

therapeutic efficacy remains to be confirmed. At present, treatment with nucleoside analogs has been applied in the treatment of ACLF-HBV and has been confirmed effective, but not all patients can survive[12-16]. When is the right time to add nucleoside analogs for treatment of ACLF-HBV patients? Therefore, it is imperative to investigate the therapeutic efficacy of comprehensive therapy in combination with antiviral treatment.

For HBeAg-negative and -positive CHB patients, there are differences in indication for treatment with nucleoside analogs, their therapeutic efficacy, and criteria for discontinuation of treatment. In the natural course of CHB, the HBV viral load significantly increases once the host immune function is compromised, which may induce acute cellular immune responses, resulting in ACLF. Therefore, maximizing long-term inhibition or elimination of HBV DNA may prevent the occurrence of ACLF in HBeAg-negative patients[17]. Nucleoside analogs can rapidly inhibit the replication of HBV and minimize propagation of the virus between hepatocytes. In addition, antiviral treatment can reduce target antigens on hepatocytes, thereby reducing attacks by cytotoxic T cells, thus attenuating the extent of damage and subsequent necrosis.

Entecavir is a cyclopentyl guanosine analog that can significantly inhibit HBV DNA polymerase, which then suppresses initiation of DNA replication and extension, resulting in reduction of viral load[18-20]. Among four commercially available nucleoside analogs, entecavir is the most potent antiviral drug, and it has the lowest frequency of resistance (1% after 4 years)[21,22]. The US Guidelines for Diagnosis and Treatment of Chronic Hepatitis B recommends entecavir as the first-line antiviral drug for treatment of patients with HBV-related liver failure[23]. Therefore, the present study aimed to investigate the efficacy of entecavir in combination with comprehensive therapy of ACLF-HBV, according to MELD score.

In 2003, Malinchoc *et al*[11] analyzed clinical information of 231 patients undergoing transjugular intrahepatic portosystemic stent shunt procedures. They applied Cox proportional hazards regression to establish the MELD score, which was then used to evaluate the risk of death among patients with liver diseases of any cause, achieving favorable reliability. A 2002 study revealed that MELD scores were given priority in making the decision to perform liver transplantation[24]. In addition, MELD score is also a sensitive indicator of the severity of liver failure.

In the present study, mean scores in the high MELD score group increased significantly before death, and mortality was 95.83% (23/24). Therapeutic failure in this group may be attributed to the fact that suppression of viral replication requires a relative long period of time; a previous study has determined that treatment with entecavir can significantly reduce the viral load within 4 wk[25]. However, treatment in this group lasted on average only 17.2 d, in the setting of fatal liver failure. In addition, ACLF pathogenesis is complex; the rapid increase in HBV replication is only one factor contributing to liver failure. Once liver failure is initiated, additional antiviral treatment is not adequate to prevent progression.

No significant differences in scores were observed before and after antiviral treatment in the intermediate MELD score group, which may account for the difference in outcome [48.15% (26/54) recovered and 51.85% (28/54) died]. Death in this group may be related to late-stage ACLF or to deteriorating liver function in the early or intermediate stages. In this CHB condition, massive liver necrosis may occur from induction of potent cellular immune responses; as a result, antiviral treatment may not be effective. Survival of 48.15% (26/54) of these patients may be attributed to the potential for liver regeneration, even if several parameters suggest liver failure. In this group, the course of treatment was 54.0 d, so HBV DNA replication was significantly suppressed, and fur-

ther liver necrosis was prevented.

In the low MELD score group, scores were significantly decreased by antiviral treatment, and the mortality was 3.23% (1/31). The majority of patients recovered, possibly because they had early-stage ACLF, a low risk for development of cellular immune responses, and the potential for compensatory liver regeneration. The mean length of treatment was 66.1 d; enough time for entecavir to accomplish an antiviral effect, which, together with effective liver regeneration, promotes recovery from liver failure.

We suggest that timely administration of an antiviral agent may improve prognosis for HBeAg-negative ACLF patients, and that prognosis can be predicted by the MELD score. When the score is $\leqslant$ 22, most patients may survive if given comprehensive therapy in combination with antiviral treatment. When the MELD is 22-30, survival, if given antiviral treatment, will depend on the severity of liver failure, the developmental trend of the disease, and whether comprehensive therapeutic interventions provide enough time for antiviral drugs to exert their effects. When the MELD score is $\geqslant$ 30, the vast majority of patients will not recover with comprehensive plus antiviral therapy. These patients are at high risk for death and liver transplantation should be considered.

COMMENTS

Background

In the natural course of chronic hepatitis B, the hepatitis B virus (HBV) viral load significantly increases once the host immune function is compromised, which may induce acute cellular immune responses, resulting in acute-on-chronic liver failure (ACLF). Therefore, maximizing long-term inhibition or elimination of HBV DNA may prevent the occurrence of ACLF in hepatitis B e antigen (HBeAg)-negative patients.

Research frontiers

In previous studies, treatment with nucleoside analogs has been used for acute-on-chronic hepatitis B liver failure (ACLF-HBV) and has been confirmed as effective. When is the right time to add nucleoside analogs to treatment of ACLF-HBV patients? The present study investigated the optimal timing, according to model for end-stage liver disease (MELD) score, for therapeutic efficacy of entecavir for ACLF-HBV in HBeAg-negative patients.

Innovations and breakthroughs

For HBeAg-negative patients with ACLF-HBV, when entecavir was added to comprehensive therapy, patients with different MELD scores may have a different outcome; a MELD score $\geqslant$ 30 predicted very poor prognosis due to fatal liver failure.

Peer review

This issue is of great interest and is offering a new method of deciding the optimal timing for therapeutic efficacy of entecavir to treat ACLF-HBV in HBeAg-negative patients according to MELD score.

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BRIEF ARTICLE

Side population cells isolated from KATO Ⅲ human gastric cancer cell line have cancer stem cell-like characteristics

Jun-Jun She, Peng-Ge Zhang, Xuan Wang, Xiang-Ming Che, Zi-Ming Wang

Jun-Jun She, Xiang-Ming Che, Department of General Surgery, First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Peng-Ge Zhang, Department of Mathematics, Xidian University, Xi'an 710061, Shaanxi Province, China
Xuan Wang, Department of Endocrinology, First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Zi-Ming Wang, Department of Urology, Second Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China
Author contributions: She JJ, Che XM, Wang ZM made substantial contributions to conception and design; She JJ performed the majority experiment and drafted the article, revised it critically for important intellectual content; Wang X performed partial experiments and revised the manuscript; Zhang PG did the data analysis; all authors approved the version to be published.
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Correspondence to: Jun-Jun She, PhD, Department of General Surgery, First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China. sjuns@sina.com
Telephone: +86-29-85323870 Fax: +86-29-88202132
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Abstract

AIM: To investigate whether the side population (SP) cells possess cancer stem cell-like characteristics *in vitro* and the role of SP cells in tumorigenic process in gastric cancer.

METHODS: We analyzed the presence of SP cells in different human gastric carcinoma cell lines, and then isolated and identified the SP cells from the KATO Ⅲ human gastric cancer cell line by flow cytometry. The clonogenic ability and self-renewal were evaluated by clone and sphere formation assays. The related genes were determined by reverse transcription polymerase chain reaction. To compare tumorigenic ability, SP and non-side population (NSP) cells from the KATO Ⅲ human gastric cancer cell line were subcutaneously injected into nude mice.

RESULTS: SP cells from the total population accounted for 0.57% in KATO Ⅲ, 1.04% in Hs-746T, and 0.02% in AGS (CRL-1739). SP cells could grow clonally and have self-renewal capability in conditioned media. The expression of *ABCG2*, *MDRI*, *Bmi-1* and *Oct-4* was different between SP and NSP cells. However, there was no apparent difference between SP and NSP cells when they were injected into nude mice.

CONCLUSION: SP cells have some cancer stem cell-like characteristics *in vitro* and can be used for studying the tumorigenic process in gastric cancer.

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Key words: Side population; Cancer stem cells; Self-renewal; Gastric cancer; KATO Ⅲ

Peer reviewers: Kazuaki Takabe, MD, PhD, Assistant Professor of Surgery and Assistant Professor of Biochemistry and Molecular Biology, Surgical Oncology, Virginia Commonwealth University Massey Cancer Center, Medical College of Virginia, Virginia Commonwealth University, PO Box 980011, Richmond, VA 23298-0011, United States; Hikaru Nagahara MD, PhD, Professor, Aoyama Hospital, Tokyo Women's Medical University, 2-7-13 Kita-Aoyama, Minatoku, Tokyo 107-0061, Japan

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INTRODUCTION

Cancer stem cells (CSC) have the characteristics of longevity, self-renewal and proliferation. It is believed that cancer stem cells are responsible for the heterogeneity and relapse of certain cancers. Flow cytometric analysis of cell surface antigens has served as the main tool to characterize CSC. CSC populations have already been identified in several solid tumors, including breast cancer[1], brain tumor[2], prostate cancer[3], melanoma[4], retinoblastoma[5], lung cancer[6], and colon cancer[7]. CSC often expresses the multi-drug resistant 1 (MDR1) or adenosine-triphosphate (ATP)-binding cassette (ABC) transporter genes, which help promote chemoresistance, a phenotype of side population (SP) cells[8].

SP cells can efflux the DNA-binding dye Hoechst33342 through an ABC membrane transporter. SP cells were initially investigated as part of the hematopoietic stem cells, and harbored stem cell-like characteristics[9]. Although conflicting results have been recently reported[10,11], most studies that investigated cell lines and tumors such as Cal-51[12], human breast carcinoma (MCF7)[13], human glioma (U373)[14], mouse ovarian carcinoma (MOVCAR 7)[15], human nasopharyngeal carcinoma (CNE-2)[16], Huh7 and PLC/PRF/5 hepatocellular carcinoma[15,16], 4T1 and NXS2 murine carcinoma[17] showed that these cell lines or tissues possessed SP cells, which were described to have stem cell-like fractions. Most studies suggested that SP cell analysis can be used to identify cancer stem cell populations[18].

Gastric cancer is the second most common cancer in the world. A total of 21 000 new cases of gastric cancer were diagnosed in the United States in 2010 with a projected five-year mortality rate exceeding 65%[19]. Aggressive surgery followed by chemotherapy resulted in a clinical response of 20%-35%. However, a majority of patients relapse and become drug-resistant. Various types of ABC transporters, especially ABC transporter genes 2 (*ABCG2*), contribute to drug resistance in cancers including gastric cancer, by pumping chemotherapeutic drugs out of cancer cells[20]. Therefore, SP cells could be used as a therapeutic target and for preventing relapse and drug-resistance in gastric cancer as well.

In this study, we analyzed the percentage of SP and non-side population (NSP) cells from several gastric cancer cell lines. By doing so, we sorted SP and NSP cells separately from the KATO Ⅲ cell lines and estimated whether the SP cell fraction possessed cancer stem cell-like characteristics *in vitro*.

MATERIALS AND METHODS

SP and NSP presentation

The human gastric cancer cell lines including KATO Ⅲ (ATCC NO: HTB-103), Hs-746T (HTB-135) and AGS (CRL-1739) were procured from American Type Culture Collection. KATO Ⅲ and Hs-746T were grown in DMEM supplemented with 100 mL/L fetal bovine serum (FBS) and penicillin/streptomycin at 37 ℃ in a humidified atmosphere with 50 mL/L CO_2. AGS was grown in RPMI-1640 supplemented with 100 mL/L FBS and penicillin/streptomycin at 37 ℃ in a humidified atmosphere with 50 mL/L CO_2. To analyze SP and NSP cell fractions, the cells were removed from their dishes with 2.5 g/L trypsin and 0.5 g/L ethylenediaminetetraacetic acid, centrifuged, washed with PBS and resuspended at 37 ℃ in Hank's balanced salt solution (HBSS) containing 20ml/L FBS. Cells (1×10^6) were labeled in HBSS with 5.0 mg/L Hoechst33342 dye (Sigma, St. Louis, MO) either alone or in combination with 50 mol/L verapamil (Sigma, St. Louis, MO) at 37 ℃ for 90 min. After washing three times with PBS, the cells were resuspended in HBSS containing 20 mL/L FBS and 1mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), passed through a 40-μm mesh filter, then maintained at 4 ℃ until flow cytometric analysis. Then, 1×10^6 viable cells were analyzed and sorted using a FACS Vantage SE Cell Sorter (BD Biosciences, CA). The Hoechst dye was excited with a UV laser and its fluorescence was measured with both 675/20 (Hoechst Red) and 424/44 filters (Hoechst Blue). The analysis was repeated three times.

Clone formation assays

Following melting 10 g/L agar (DNA grade) in the microwave and warming 2 × Dulbecco's modified eagle medium (DMEM) supplemented with 200 mL/L FBS to 40 ℃ in a water bath, equal volumes of the two solutions were mixed to yield a new solution of 5 g/L agar, 1 × DMEM and 100 mL/L FBS. Next, 1.0 mL of mixed solution was added to each well of a 6-well plate to form the base agar. Then, 7 g/L agar (DNA grade) was melted in the microwave and cooled to 40 ℃ in a water bath; similarly, 2 × DMEM and 200 mL/L FBS were warmed to the same temperature. Freshly sorted SP and NSP cells from KATO Ⅲ were passed through a 40-μm filter to provide a single cell suspension and were counted. Three mL DMEM, 1.5 mL 2 × DMEM containing 200 mL/L FBS and 1.5 mL agar including 400 cells were then mixed together, and a 1.5 mL cell suspension of this solution was placed into each well of a 6-well plate as the top agar. Finally, 100 cells were seeded into each well, and were incubated at 37 ℃ in a humidified incubator for 2-3 wk. Colonies were either left unstained, or were stained with 5 g/L MTT (Sigma, St. Louis, MO) for no more than one hour, and counted under a dissecting microscope. The procedure was repeated three times. After SP cells were seeded in soft agar assays, colonies containing more than 50 cells (primary colony) were removed from soft agar with sterile Pasteur pipettes, treated with trypsin and mechanically dissociated into single cells. Then, 100 cells were seeded into each well at 37 ℃ in a humidified incu-

bator for another 2-3 wk. Colonies (secondary colony) were stained with 5 g/L MTT for no more than one hour, and counted under a dissecting microscope. The procedure was repeated three times.

Lentivirus infection

Plasmid DNA was kept at a 3:3:1 ratio [Vector (EGFP): Δ8.91: VSV-G] according to the lipofectamine protocol (Invitrogen). Next, 2.5 μg plamid DNA was diluted in 500 μL serum-free DMEM for each well, and 2.5 μL Mix PLUS reagent and 6.25 μL Lipofectamine™ LTX reagent were added to the diluted DNA and incubated for 30 min at room temperature. Then, approximately 500 μL of the DNA-Lipofectamine™ LTX complex was added to each well containing 293FT cells, which were 40%-50% confluent. The cells were incubated at 37 ℃ in a CO_2 incubator for six hours and the medium was changed for transfected cells. After 24 h, the media from 293FT cells was collected, and 8 mg/L polybrene was added and transferred to target cells. The transfer of media from 293 FT to KATO Ⅲ cells was repeated after 24 h and 48 h.

Sphere formation assays

To test sphere formation in suspension, sorted SP and NSP cells from KATO Ⅲ were passed through a 40-μm filter to provide a single cell suspension. Next, 4 mL medium containing 100 cells were added to the 6-well plates with serum-free media including F12 with EGF (10 μg/L), insulin (20 mg/L) and basic fibroblast growth factor (bFGF) (10 μg/L). Aliquots of epidermal growth factor (EGF), insulin and bFGF were added twice a week. After 10-14 d, plates were visually assayed for the formation of floating spheres. To assess the ability of primary spheres to form secondary spheres, colonies containing more than 30 cells were collected by centrifugation and trypsinized to yield single cells. After passing through a 40-μm filter, 4 mL serum-free media containing 100 cells were added to each 6-well plate and were cultured 10-14 d for secondary sphere formation. Spheres were counted under a dissecting microscope.

Gene expression detection

1×10^5 SP and NSP cells from the KATO Ⅲ total population were collected in a separate centrifuge tube with 350 μL RLT buffer containing 10 g/L 2-mercaptoethanol, total RNA was extracted from these cells using a RNeasy Mini Kit (Qiagen, CA) according to the protocol provided by the manufacturer. RNA was transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen, CA). RT-PCR was performed using a SuperScript One-Step kit (Invitrogen, CA). The primers were as follows: Glyceraldehyde-3-phosphate dehydrogenase, 5-CTG CAC CAC CAA CTG CTT AG-3 and 5-AGG TCC ACC ACT GAC ACG TT-3; *ABCG2*, 5-GGG TTC TCT TCT TCC TGA CGA CC-3 and 5-TGG TTG TGA GAT TGA CCA ACA GAC C-3; *MDR1*, 5-GCC TGG CAG CTG GAA GAC AAA TAC-3 and 5-ATG GCC AAA ATC ACA AGG GTT AGC-3; B-cell-specific Moloney murine leukemia virus insertion site 1 (*Bmi-1*), 5-AGC AGA AAT GCA TCG AAC AA-3 and 5-CCT AAC CAG ATG AAG TTG CTG A-3; Octamer-binding transcription factor 4 (*Oct-4*), 5-GAG AAT TTG TTC CTG CAG TGC-3 and 5-GTT CCC AAT TCC TTC CTT AGT G-3. The PCR products were separated by electrophoresis on a 20 g/L agarose gel.

Tumor formation assays

Sorted SP and NSP cells from KATO Ⅲ were resuspended in 50 μL HBSS, ranging in density from 10^4 cells to 10^3 cells, and then mixed with 50 μL Matrigel (Becton Dickinson, NJ) to prevent injected cell dispersion and loss. Then, cells were subcutaneously injected into 6- or 7-wk old nude mice on the day of sorting. Nude mice were obtained from the Animal Institute of the Xi'an Jiaotong University, China (XJTU). All experiments were approved by the Animal Care Committee of XJTU. Mice were monitored every day to assess tumor formation for 6-8 wk after transplantation.

Statistical analysis

All values were expressed as mean ± SD. Any significant difference among mean values was further evaluated by the Student's *t* test.

RESULTS

Identification of SP in gastric cancer cell lines

SP was identified through their fluorescence profile in a dual-wavelength analysis by flow cytometry (Hoechst red 675/20; Hoechst blue 424/44), and were shown as a characteristic tail separated from the complete population.

The percentage of SP cells in human gastric cancer cell lines was 0.57% in KATO Ⅲ, 1.04% in Hs-746T, and 0.02% in AGS (Figure 1). SP cells decreased in number following treatment with verapamil, an inhibitor of the ABC transporter. To further investigate the function of SP and NSP cells in these gastric cancer cell lines, *in vitro* and *in vivo* tumorigenicity was studied mainly among KATO Ⅲ cells.

Clone formation

The clonogenic ability of SP and NSP cells were examined when seeded as single cells. The clonogenic efficiency of SP cells was significantly higher than NSP cells in soft agar assays (Figure 2A and B). Moreover, most SP cells could divide into colonies of more than 50 cells in soft agar after three weeks. There was no apparent difference between NSP cells and the complete population. The self-renewal ability of SP cells was also examined in serial soft agar assays. There was an increase in clonogenic efficiency from primary to secondary colonies. Moreover, secondary colonies were similar in size to the primary colonies, thus demonstrating that SP cells can maintain and expand themselves in serial soft agar assays.

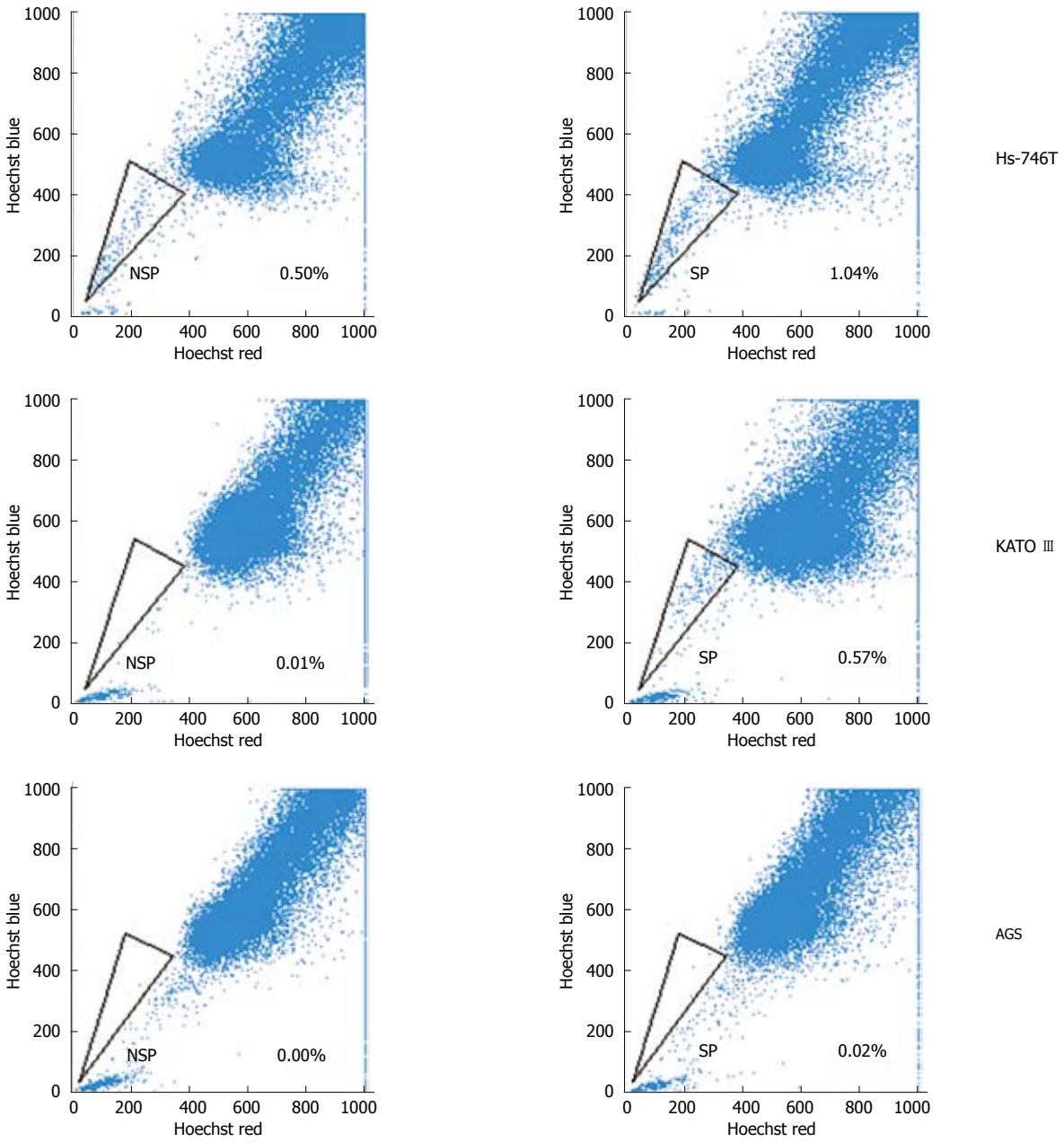


Figure 1 Side population and non-side population cells from several human gastric cancer cell lines were analyzed through uptake of the DNA binding dye Hoechst33342 with or without the presence of verapamil. NSP: Non-side population; SP: Side population.

To ascertain whether the colony comes from a single cell, KATO Ⅲ was infected with enhanced green fluorescent protein-expressing (EGFP) lentivirus and equal numbers of KATO Ⅲ-GFP and KATO Ⅲ cells were seeded from primary to second passage into serial soft agar assays. It was revealed that KATO Ⅲ-GFP cells expressed strong green fluorescence and showed a similar growth rate compared to non-infected KATO Ⅲ. Moreover, there were no mixed colonies (Figure 2C), indicating that the colonies came from single cells.

Sphere formation

The ability of SP and NSP cells to generate spherical clones and self-renewal was evaluated by sphere formation assays (Figure 3A). It was difficult to observe floating spheres from NSP cells (Figure 3B). SP cells started to form floating spheres after 2-3 d of seeding, and became primary spheres of more than 30 cells after 10-14 d (Figure 3C). The secondary sphere occurred slightly more quickly and frequently than the primary spheres, suggesting that SP cells have self-renewal ability.

Gene expression

First, the expression of ABC transporters, including *ABCG2* and *MDR1*, was observed, which was significantly higher in SP than in NSP cells (Figure 4). To further

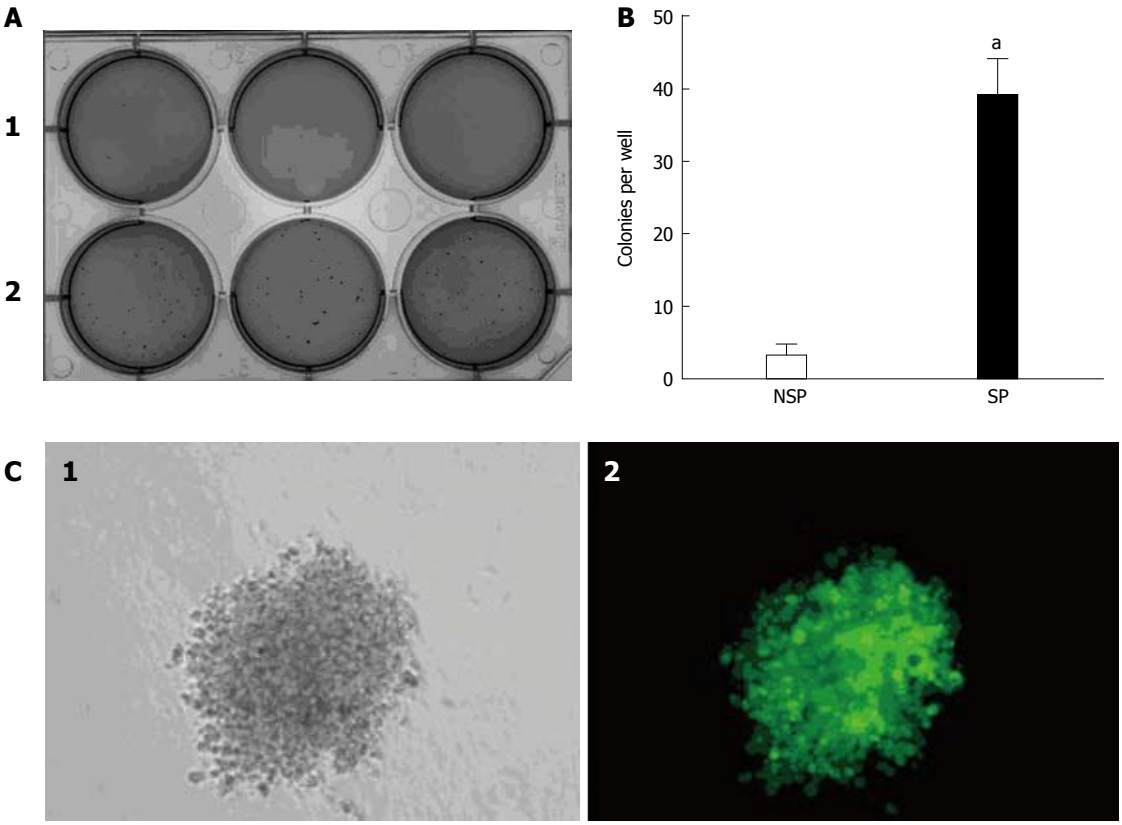


Figure 2 Soft agar assays and fluorescence for KATO Ⅲ-green fluorescent protein cells. A: Side population (SP) cells (A-2) were more clonogenic than non-side population (NSP) cells (A-1); B: The assay was repeated 3 times, and the column diagram indicated that there was a statistically significant difference in clonogenic efficiency between SP and NSP cells ($^aP < 0.05$ *vs* NSP cells); C: KATO Ⅲ-green fluorescent protein (GFP) cells expressed strong green fluorescence in regular culture, with no mixed colonies resulting from a single KATO Ⅲ-GFP cell. C-1 was observed under white light, while C-2 was observed under fluorescent light.

determine whether SP cells have stem cell-like characteristics, SP and NSP cells were examined for their expression of stem cell markers. The results demonstrated that the expression of *Oct-4* and *Bmi-1* in SP cells was higher than in NSP cells (Figure 4), suggesting that SP cells had some of the characteristics of undifferentiated stem cells.

Tumor formation

To compare tumorigenic ability, SP and NSP cells from the KATO Ⅲ human gastric cancer cell line were subcutaneously injected into the nude mice. Each mouse was individually injected with different amounts of SP and NSP cells. Six weeks after injection, although tumor diameter was smaller in NSP than in SP cells, four out of six mice could form tumors when injected either with SP or NSP cells (Table 1). All remaining mice could form tumors after eight weeks.

DISCUSSION

To our knowledge, there has been no report identifying SP cells from the gastric cancer cell lines. As such, in this paper we analyzed SP and NSP cells in the KATO Ⅲ, Hs-746T and AGS human gastric cancer cell lines. In the KATO Ⅲ and Hs-746T cell lines, which have a high tumorigenic ability, SP cells accounted for 0.57% and 1.04% of the total population, moreover, they occupied 0.02% in the high tumorigenic AGS cell line. Therefore, it is challenging to draw a conclusion from the percentage of SP cells in different cell lines to compare their tumorigenic ability. These data are consistent with the C6 glioma cell line, which was mainly composed of cancer stem cells, although many of these cells were neither CD133+ nor a side population[21]. Further studies are therefore needed to compare the relationship between SP prevalence and tumorigenicity.

Single cells that grow anchorage-independently in soft agar are known to be malignant. Thus, cell lines should contain those cells which could form colonies and those that did not form colonies in soft agar assays. As such, soft agar assays can be used to investigate this tumorigenic ability *in vitro*. By seeding the mixed cells of KATO Ⅲ-GFP and KATO Ⅲ into soft agar, we could conclusively determine that colonies came from single cells. The clonogenic ability of SP cells isolated from KATO Ⅲ was higher than that of NSP cells in soft agar and sphere forming assays. This is in agreement with the reports for the MCF7[13] and ARO[11] cell lines. In original animal transplant experiments, SP of KATO Ⅲ were injected into nude mice subcutaneously, ranging in density from 10^4 to 10^2 cells. The tumor forms in mice by injecting 10^4 (2 out of 2) and 10^3 cells (2 out of 2) SP in 6 wk. The other mice did not form tumor by injecting 5×10^2 (0 out of 2) and 1×10^2 cells (0 out of 2) SP until week

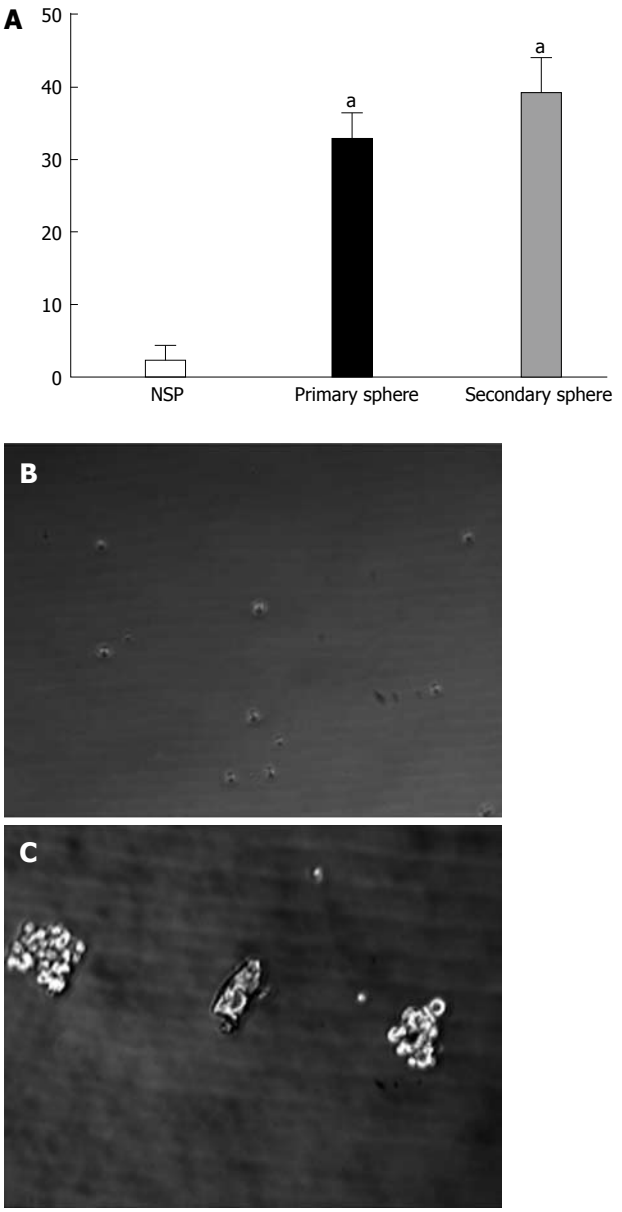


Figure 3 Serial sphere assays. A: The assay was repeated 3 times, and the column diagram indicated a statistically significant difference in clonogenic efficiency between non-side population (NSP) and side population (SP) cells in primary and secondary sphere assays ($^aP < 0.05$ *vs* NSP cells); however, there was no significant difference between primary and secondary sphere assays; B: Sphere assay for NSP cells; C: Sphere assay for SP cells.

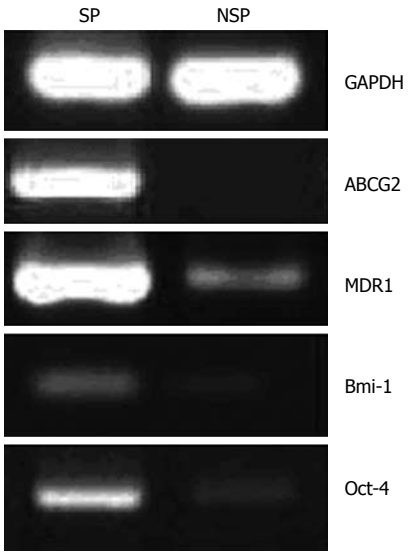


Figure 4 Glyceraldehyde-3-phosphate dehydrogenase, adenosine-triphosphate-binding cassette sub-family G member 2, multidrug resistance protein 1, B-cell-specific Moloney murine leukemia virus insertion site 1 and octamer-binding transcription factor 4 RNA expression of side population and non-side population cells isolated from KATO Ⅲ by reverse transcription polymerase chain reaction. SP: Side population; NSP: Non-side population; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ABCG2: Adenosine-triphosphate-binding cassette sub-family G member 2; MDR1: Multidrug resistance protein 1; Bmi-1: B-cell-specific Moloney murine leukemia virus insertion site 1; Oct-4: Octamer-binding transcription factor 4.

Table 1 Tumor diameters in side population and non-side population cells sorted from the KATO Ⅲ cell line in nude mice after 6 wk

Mouse	Cell	Cell dose	Tumor diameter (cm)
A	SP	1×10^4	2.0
		1×10^3	1.0
	NSP	1×10^4	2.0
		1×10^3	0.5
B	SP	1×10^4	2.4
		1×10^3	1.3
	NSP	1×10^4	2.0
		1×10^3	Not tested
C	SP	1×10^4	2.0
		1×10^3	1.1
	NSP	1×10^4	2.0
		1×10^3	0.9
D	SP	1×10^4	2.2
		1×10^3	1.2
	NSP	1×10^4	1.9
		1×10^3	1.0
E	SP	1×10^4	1.8
		1×10^3	1.2
	NSP	1×10^4	1.8
		1×10^3	Not tested
F	SP	1×10^4	2.0
		1×10^3	1.0
	NSP	1×10^4	1.8
		1×10^3	1.0

SP: Side population; NSP: Non-side population.

10. In tumor formation assays, SP and NSP of KATO Ⅲ ranging from 10^4 to 10^3 cells were injected. In our study, 12 injection sites for SP cells could form tumors in six weeks. However, 12 injection sites for NSP cells could also form tumors as well, although the observation period had to be increased to eight weeks. There was no significant difference in tumorigenicity *in vivo* between SP and NSP cells, which suggested that although *CSC* was enriched in SP cells, it may contain both SP and NSP cells.

Indefinite self-renewal is one of the essential properties of stem cells. A stem cell could undergo asymmetric division continuously, producing one cell that retains self-renewal ability and differentiates into a mature cell. Stem cells and progenitor cells have the property of

anchorage-independent growth[22]. Therefore, soft agar and sphere formation assays can be used to test the self-renewal ability of stem cells *in vitro*[23,24]. In serial soft agar and sphere formation assays, single cells isolated from primary colonies coming from SP cells could form a secondary colony, suggesting that SP cells have the self-renewal capacity.

Bmi-1 is a transcriptional repressor belonging to the polycomb group of transcription factors. It has been shown to be important in the self-renewal of both normal and leukemic stem cells, as well as neuronal stem cells[25]. Oct-4 is a POU homeodomain transcription factor that is a key regulator of self-renewal in embryonic stem cells[26]. Cellular expression of Oct-4 is believed to have the capacity for self-renewal[27]. Moreover, the self-renewal ability of cancer stem cells could drive tumorigencity[28]. Oct-4 and Bmi-1 overexpression in SP cells isolated from KATO Ⅲ was observed, which might reflect the property of self-renewal of SP cells.

While ABCG2 can efflux the DNA binding dye Hoechst 33342, which could induce an SP phenotype, MDR1 is the best-studied member of the ABC transporter superfamily of genes[29]. The expression level of *ABCG2* and *MDR1* mRNA was higher in SP than in NSP cells as determined by RT-PCR analysis, which contributes to the chemotherapeutic resistance of SP cells, and thus may be a target for cancer therapy.

COMMENTS

Background

Gastric cancer is the second leading cause of cancer-related deaths worldwide. Cancer stem cells have the characteristics of longevity, self-renewal and proliferation. It is believed that cancer stem cells are responsible for the heterogeneity and relapse of certain cancers including gastric cancer.

Research frontiers

Side population (SP) cells can efflux the DNA-binding dye Hoechst33342 through an adenosine-triphosphate-binding cassette (ABC) membrane transporter and harbor stem cell-like characteristics. Recent studies suggested that SP cell analysis can be used to identify cancer stem cell populations. However, there has been no report identifying SP cells from the gastric cancer cell lines.

Innovations and breakthroughs

This is the first study to analyze SP and non-side population (NSP) cells in several human gastric cancer cell lines. The clonogenic ability of SP cells isolated from KATO Ⅲ was higher than that of NSP cells in soft agar and sphere forming assays. *Oct-4* and *Bmi-1* overexpression in SP cells isolated from KATO Ⅲ might reflect the property of self-renewal of SP cells. Moreover, *ABCG2* and *MDR1* overexpression contributes to the chemotherapeutic resistance of SP cells, and thus may be a target for cancer therapy.

Applications

SP cells could be used as a therapeutic target and for preventing relapse and drug-resistance in gastric cancer.

Terminology

Cancer stem cells are characterized by its self-renewal capacity, differentiation potential, and cancer-initiating ability. Side population cells expressing the ABC transporter were distinguished from whole cell population. Recent studies demonstrated that SP cells could be characterized as cancer stem cells in primary tissues and tumor cell lines, therefore, it was postulated to be responsible for tumor development and recurrence.

Peer review

Overall, this paper is very well written with good English and solid methodology. The biggest issue is that the content is not new. Basically, what the authors did was to confirm and characterize the side population cells in gastric cancer cell lines, which is not surprising. With that said, it is believed no one has done it in these cells, and it still may somewhat be informative to readers.

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BRIEF ARTICLE

7-difluoromethoxyl-5,4′-di-n-octylgenistein inhibits growth of gastric cancer cells through downregulating forkhead box M1

Hong-Lin Xiang, Fei Liu, Mei-Fang Quan, Jian-Guo Cao, Yuan Lv

Hong-Lin Xiang, Fei Liu, Mei-Fang Quan, Jian-Guo Cao, Yuan Lv, Laboratory of Medical Engineering, Medical College, Hunan Normal University, Changsha 410013, Hunan Province, China

Author contributions: Xiang HL, Liu F, Quan MF and Lv Y performed the majority of experiments; Cao JG designed the study and wrote the manuscript.

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Correspondence to: Jian-Guo Cao, Professor, Laboratory of Medical Engineering, Medical College, Hunan Normal University, Changsha 410013, Hunan Province, China. caojianguo2005@yahoo.com.cn
Telephone: +86-731-8912434 Fax: +86-731-8912417

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Abstract

AIM: To investigate whether the 7-difluoromethoxyl-5, 4′-di-n-octylgenistein (DFOG), a novel synthetic genistein analogue, affects the growth of gastric cancer cells and its mechanisms.

METHODS: A series of genistein analogues were prepared by difluoromethylation and alkylation, and human gastric cancer cell lines AGS and SGC-7901 cultured *in vitro* were treated with various concentrations of genistein and genistein analogues. The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were incubated by DFOG at different concentrations. The growth inhibitory effects were evaluated using MTT and clonogenic assay. The distribution of the phase in cell cycle was analyzed using flow cytometric analysis with propidium iodide staining. The expression of the transcription factor forkhead box M1 (FOXM1) was analyzed by reverse transcription-polymerase chain reaction and Western blotting. The expression levels of CDK1, Cdc25B, cyclin B and $p27^{KIP1}$ protein were detected using Western blotting.

RESULTS: Nine of the genistein analogues had more effective antitumor activity than genistein. Among the tested analogues, DFOG possessed the strongest activity against AGS and SGC-7901 cells *in vitro*. DFOG significantly inhibited the cell viability and colony formation of AGS and SGC-7901 cells. Moreover, DFOG efficaciously arrested the cell cycle in G2/M phase. DFOG decreased the expression of FOXM1 and its downstream genes, such as CDK1, Cdc25B, cyclin B, and increased $p27^{KIP1}$ at protein levels. Knockdown of FOXM1 by small interfering RNA before DFOG treatment resulted in enhanced cell growth inhibition in AGS cells. Up-regulation of FOXM1 by cDNA transfection attenuated DFOG-induced cell growth inhibition in AGS cells.

CONCLUSION: DFOG inhibits the growth of human gastric cancer cells by down-regulating the FOXM1 expression.

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Key words: Gastric cancer; 7-difluoromethoxyl-5,4′-di-n-octylgenistein; Genistein; Forkhead box M1; Therapeutic action

Peer reviewers: Soon-Sun Hong, Professor, Inha University School of Medicine, Clinical Research Center, 2F, Jeongsuk Bld, 3-ga, Shinheung-dong, Incheon 400-712, South Korea; Hikaru Nagahara, Professor, Department of Gastroenterology, Aoyama Hospital, Tokyo Women's Medical University, 2-7-13 Kitaaoyama, Minato-ku, Tokyo 107-0061, Japan

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INTRODUCTION

Genistein, 5,7,4'-trihydroxylisoflavone, as one of the active constituents of soybean products, has been reported to possess anti-cancer activities[1,2]. But probably because of its low solubility in both water and organic solvent, genistein has a very low bioavailability. The introduction of the fluorine or fluoride-bearing alkyl such as CF_3 or HCF_2 into organic molecules dramatically changes their physiological, physical and chemical properties[3]. To our best knowledge, the introduction of fluorine moiety into the aryl part of the flavonoid molecule can enhance their biological activities, including anti-bacterial, anti-fungal and anti-viral activities[3]. Fluorinated 3, 4-dihydroxychalcones have illustrated interesting biological activities, including anti-peroxidation and antitumor activity *in vitro*[4]. We have previously reported that introduction of CF_3 or HCF_2 group into chrysin (5,7-dihydroxylflavone) molecule can improve their anticancer activities[5-7]. Recently, we synthesized a series of difluoromethoxylated genistein analogues and determined their protective effects against vascular endothelial cells[8]. However, there are few studies which reported the anti-cancer effect of fluorinated genistein analogues.

Gastric cancer is one of the most common malignancies in the world and its incidence and mortality rank first in China[9]. Recent data indicate that the mortality of gastric cancer in China tends to increase and it severely threatens the health and life of people[10]. At present, surgery and chemotherapy remain as the main modalities in the management of gastric cancer, but the curative effect of the existing chemotherapeutic drugs is not satisfactory, which cause numerous side effects. Therefore, it has been a focus to search for new drugs capable of preventing and treating gastric cancer and other malignancies. Gastric cancer has been shown to have activated forkhead box protein M1 (FOXM1) signaling pathway[11]. The FOXM1 belongs to a family of evolutionary conserved transcriptional regulators that were characterized by the presence of a DNA-binding domain called the forkhead box or winged helix domain[12]. It has been shown that FOXM1 signaling plays an important role in cellular developmental pathways, and activation of FOXM1 signaling is associated with carcinogenesis[13]. FOXM1 signaling is frequently up-regulated in cancers, including lung, breast, pancreatic and gastric cancer[11,14-16]. Moreover, FOXM1 has been shown to regulate transcription of cell cycle genes, including Cdc25B, CDK1, cyclin B and $p27^{KIP1}$[13,17]. Recently, it has been reported that FOXM1 expression could serve as an independent predictor of a poor survival of gastric cancer patients[11]. Studies by Wang *et al*[18] have shown that genistein may inhibit FOXM1 activation in pancreatic cancer cells, leading to apoptotic cell death. Therefore, it is believed that the targeted inactivation of FOXM1 could represent a promising strategy for the development of novel selective anti-cancer therapies.

In the present study, we investigated whether the growth inhibitory effects of genistein and the novel synthetic genistein analogue 7-difluoromethoxyl-5,4'-di-n-octylgenistein (DFOG) on gastric cancer cells could be attributed to modulation of FOXM1 activity. We found that DFOG and genistein down-regulated the FOXM1 expression and its downstream genes, including cdc25B, CDK1, cyclin B and up-regulated $p27^{KIP1}$, resulting in the growth inhibition of gastric cancer cells. These results provide strong evidences to support that FOXM1 is a rational target in gastric cancer, and the targeted inactivation of FOXM1, especially by genistein and its analogue DFOG, may provide new insight into the strategy development for better prevention of tumor progression and/or treatment of gastric cancer.

MATERIALS AND METHODS

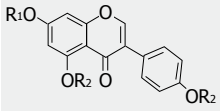
Cell culture and reagents

Human gastric cancer cell lines AGS and SGC-7901 were purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China). Cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 mg/L penicillin, and 100 mg/L streptomycin, and incubated in a humidified atmosphere of 5% CO_2 at 37 ℃. The difluoromethoxylated genistein analogues 2,4a-4h were prepared by the method described elsewhere[8] (Table 1). Primary antibodies for FOXM1, CDK1, cyclin B, $p27^{KIP1}$ and Cdc25B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Anti-β-actin antibody and Horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody were purchased from Santa Cruz Biotechnology. Lipofectamine 2000 was purchased from Invitrogen. Protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other chemicals were obtained from Sigma (St. Louis, MO, United States). Genistein (Sigma) and the difluoromethoxylated genistein analogues were dissolved in dimethyl sulfoxide (DMSO) to make a 10 mmol stock solution and were added directly to the media at different concentrations before use.

MTT assay

Cells were seeded in a 96-well plate at a density of 5000 cells/well as described previously[19]. After incubation for 24 h to allow cell attachment, different concentrations of genistein and genistein analogues (0.1, 0.3, 1.0, 3.0, 10.0 and 30.0 μmol/L) were added to each well and cultured for 24 h. Medium was removed and then incubated with 5.0 mg/mL MTT for 4 h. Then supernatant was removed after centrifugation. Finally, 100 μL of DMSO was added and absorbance at 570 nm wavelength (A_{570}) was measured by means of an Enzyme-labeling instrument (ELX-800 type; Bio-Tek, Shanghai, China). Relative cell proliferation inhibition rate = (1 - average A_{570} of the experimental group/average A_{570} of the control group) × 100%. The IC_{50} (defined as the drug concentration which 50% cell viability was inhibited) was as-

Table 1 Structures of genistein and its difluoromethylated derivatives



	Compound	R1	R2
1	Genistein (5,7,4′-trihydroxylisoflavone)	H	H
2	7-difluoromethyl genistein	CHF_2	H
4a	7-difluoromethyl-5,4′-dimethyl genistein	CHF_2	CH_3
4b	7-difluoromethyl-5,4′-diethyl genistein	CHF_2	CH_3CH_2
4c	7-difluoromethyl-5,4′-di-n-propyl genistein	CHF_2	n-C_3H_7
4d	7-difluoromethyl-5,4′-di-benzyl genistein	CHF_2	$C_6H_5CH_2$
4e	7-difluoromethyl-5,4′-diheptyl genistein	CHF_2	n-C_7H_{15}
4f	7-difluoromethyl-5,4′-di-n-octyl genistein	CHF_2	n-C_8H_{17}
4g	7-difluoromethyl-5,4′-didecyl genistein	CHF_2	n-$C_{10}H_{21}$
4h	7-difluoromethyl-5,4′-diisobutyl genistein	CHF_2	iso-C_4H_9

sessed from the dose-response curves using GraphPad Prism program (Version 4, GraphPad Software).

Clonogenic assay

Cells were plated in 24-well plates at a density of 300 cells/well for 24 h, prior to the addition of various concentrations of DFOG (1, 5 and 10 μmol/L) and 10 μmol/L genistein. After 48 h of treatment, the drug-containing medium was removed and replaced with complete growth medium. Medium was changed every 3 d for 8-10 d until visible colonies formed. Colonies were simultaneously fixed and stained with 0.5% crystal violet in methanol, and manually counted. Individually stained colonies in each well were counted and the colony formation fraction was calculated as follows: colony number/(number of cells seeded × plating efficiency), where plating efficiency is equivalent to the colony number divided by the number of cells seeded in the drug-free medium.

Cell cycle analysis by flow cytometry

Cells were plated in 6-well plates at a density of 1 000 000 cells/well for 24 h, prior to the addition of various concentrations (1, 5 and 10 μmol/L) of DFOG and 10 μmol/L genistein. After 24 h of treatment, cells were harvested, and DNA content was stained for 15 min at 37 ℃ with a solution containing 0.4% Triton X-100 (Sigma), 50 μg/mL of propidium iodide (Sigma), and 2 μg/mL of DNase-free RNase (Roche, United States). The cells were then analyzed for cell cycle perturbation using a FACSCalibur (FACS 420, Becton Dickinson, United States). The CellQuest program was used to quantitate the distribution of cells in each cell cycle phase: G1, S and G2/M.

Reverse transcription-polymerase chain reaction

Total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD, United States). The integrity of the RNA was checked by 2% agarose gel electrophoresis. Approximately 2 μg RNA was reversely transcribed following the protocol of the Super Script™ first-strand synthesis system (Invitrogen Corporation, Carlsbad, CA, United States). The cDNAs encoding FoxM1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified by polymerase chain reaction (PCR) as follows: denaturation at 94 ℃ for 30 s, annealing at 63 ℃ for 30 s and elongation at 72 ℃ for 45 s. Primer sequence was designed using Primer. For FoxM1, the forward primer was 5'-AACCGCTACTTGACATTGG-3' and reverse primer 5'-GCAGTGGCTTCATCTTCC-3'. A housekeeping gene, GAPDH was used as the internal control. The forward primer was 5'-ACCCAGAAGACTGTGG ATGG-3', and the reverse primer was 5'-TGCTGTAGCCAAATTCGTTG-3'. PCR products were analyzed by agarose (2%) gel electrophoresis.

Plasmids and transfections

FOXM1 small interfering RNA (siRNA) and control siRNA were obtained from Santa Cruz Biotechnology. The FOXM1 cDNA plasmid was purchased from OriGene Technologies Inc (Rockvile, MD, United States). Human gastric cancer AGS cells were transfected with FOXM1 siRNA and cDNA, respectively, using Lipofectamine 2000 (Invitrogen) as described by Wang *et al*[20].

Western blotting analysis

Western blotting analysis was carried out as previously described[21]. Cells were lysed in lysis buffer by incubation for 20 min at 4 ℃. The protein concentration was determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA, United States). Total proteins were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto Polyvinylidene fluoride membrane (Millipore, United States). Anti-FOXM1, anti-CDK1, anti-cyclin B, anti-$p27^{KIP1}$, anti-cdc25B and anti-β-actin rabbit polyclonal antibodies were used as primary antibodies. The signals were detected using an ECL Advance Western blotting analysis system (Amersham Pharmacia Biotech Inc., Piscataway, NJ, United States).

Statistical analysis

The SPSS 15.0 software package (SPSS Inc, Chicago, IL, United States) was used for the statistical analysis. Data were expressed as mean ± SD. The means of multiple groups were compared with one-way analysis of variance, after the equal check of variance, and the pairwise comparisons among the means were performed using the least significant difference method. Statistical comparison was also performed with two-tailed *t* test when appropriate. A $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of genistein and genistein analogues on the cell viability of gastric cancer cells

First, we examined the effects of genistein and the genistein analogues on the viability of AGS and SGC-7901

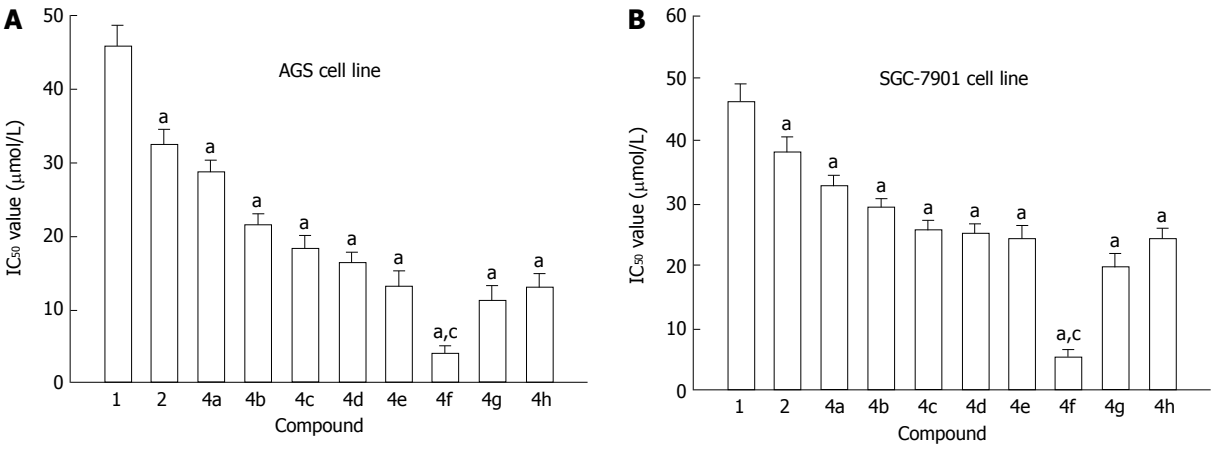


Figure 1 Inhibition of cell viability by genistein and genistein analogues. A: AGS cell line; B: SGC-7901 cell line. $^{a}P < 0.05$ *vs* treatment with genistein; $^{c}P < 0.05$ *vs* treatment with genistein or other genistein analogues. 1: Genistein (5,7,4'-trihydroxylisoflavone); 2: 7-difluoromethyl genistein; 4a: 7-difluoromethyl-5,4'-dimethyl genistein; 4b: 7-difluoromethyl-5,4'-diethyl genistein; 4c: 7-difluoromethyl-5,4'-di-n-propyl genistein; 4d: 7-difluoromethyl-5,4'-di-benzyl genistein; 4e: 7-difluoromethyl-5,4'-diheptyl genistein; 4f: 7-difluoromethyl-5,4'-di-n-octyl genistein; 4g: 7-difluoromethyl-5,4'-didecyl genistein; 4h: 7-difluoromethyl-5,4'-diisobutyl genistein.

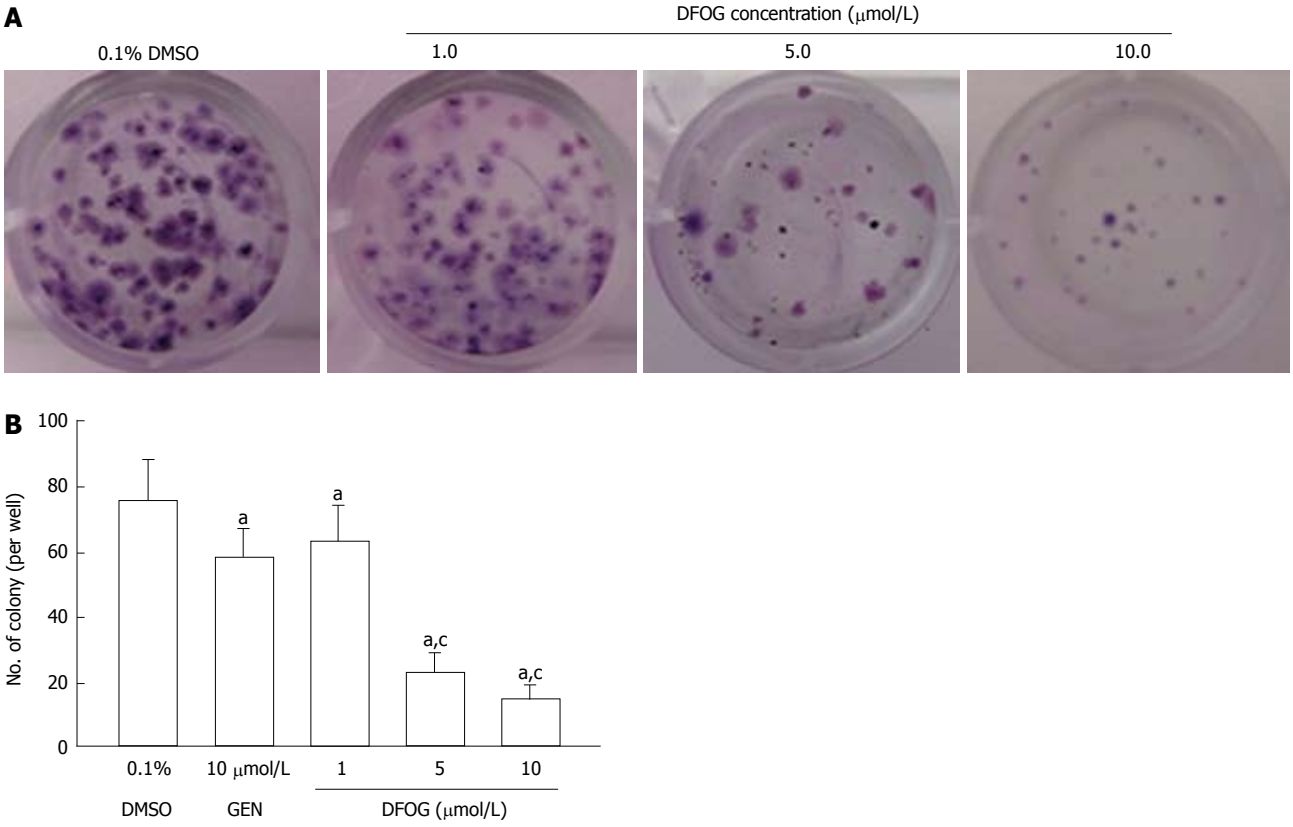


Figure 2 Decrease of colony number and inhibition of colony formation by 7-difluoromethoxyl-5,4'-di-n-octylgenistein. A: Decrease of colony number by 7-difluoromethoxyl-5,4'-di-n-octylgenistein (DFOG); B: Inhibition of colony formation by DFOG and genistein in AGS cell line. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with 10 μmol/L genistein (GEN) or 1 μmol/L DFOG.

cells using MTT assay. Nine of difluoromethylated genistein analogues had higher effective antitumor activities than genistein. Among the aforementioned analogues, DFOG showed the strongest activity against AGS and SGC-7901 *in vitro* (Figure 1A and B). IC_{50} of DFOG was 3.9 μmol/L for AGS cells and 5.2 μmol/L for SGC-7901 cells, the potency of DFOG was 11.7 and 8.9 as much as that of the lead compound, genistein (IC_{50} was 45.9 μmol/L for AGS cells and 46.3 μmol/L for SGC-7901 cells).

Effects of DFOG on the colony formation of gastric cancer cells

Next, we tested the effects of DFOG on cell growth by clonogenic assay. DFOG treatment resulted in a significant inhibition of colony formation of AGS cells

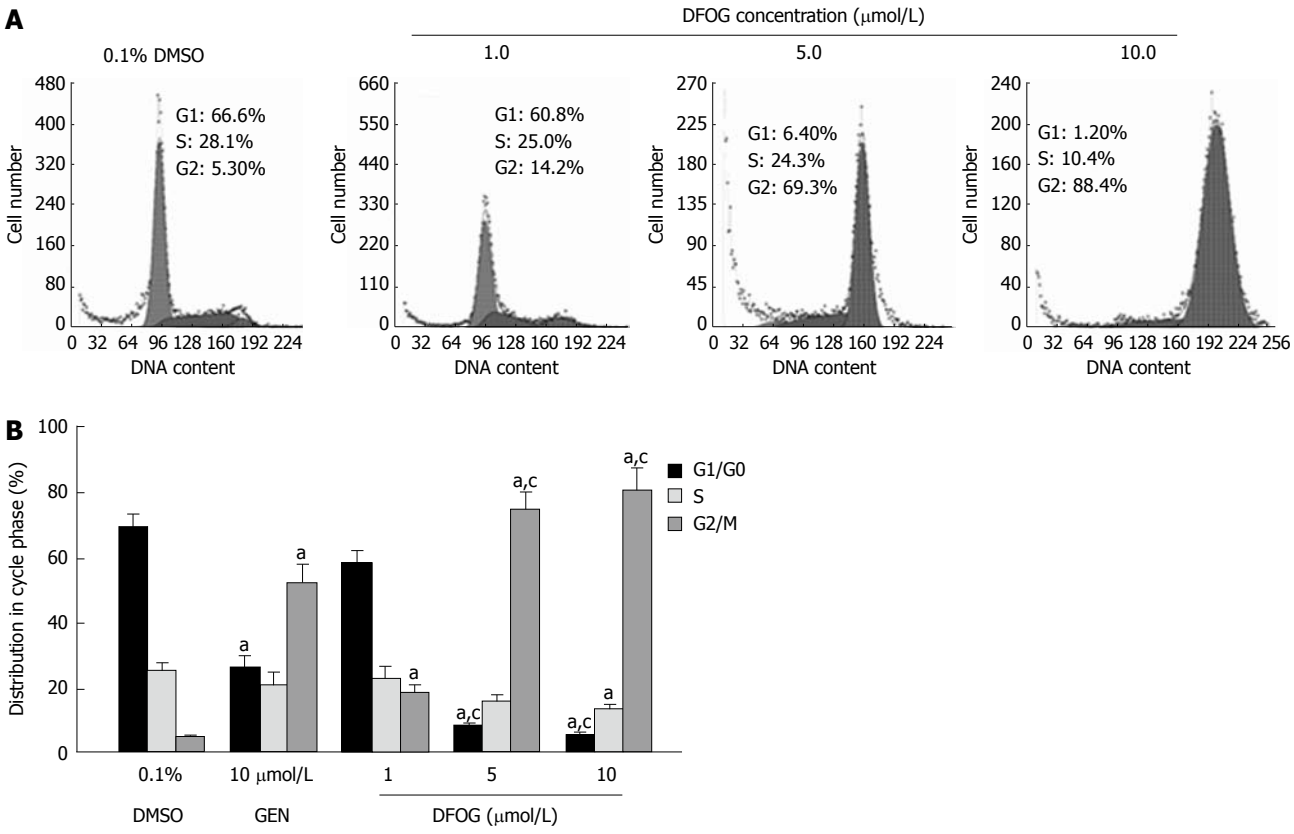


Figure 3 Increase of cells in G2/M phase and induction of cell cycle arrest in G2/M phase by 7-difluoromethoxyl-5,4'-di-n-octylgenistein. A: Increase of cells in G2/M phase by 7-difluoromethoxyl-5,4'-di-n-octylgenistein (DFOG); B: Induction of cell cycle arrest in G2/M phase by DFOG and genistein in AGS cell line. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with 10 μmol/L genistein (GEN) or 1 μmol/L DFOG.

compared with controls (Figure 2A and B). Similar results were observed in SGC-7901 cells (data not shown). These data suggests that DFOG inhibits the growth of gastric cancer cells.

Effects of DFOG on the distribution of cell cycle phase in gastric cancer cells

To assess whether the loss of cell survival could in part be attributed to the induction of cell cycle arrest, we evaluated the effects of DFOG treatment on the distribution in cell phase using flow cytometry with propidium iodide staining. As shown in Figure 3A and B, in gastric cancer cell line AGS, DFOG treatment caused a significant accumulation of cells in the G2/M phase and a marked decrease in the G1/G0 phase compared with control cells. Similar results were observed in SGC-7901 cells (data not shown). These results provided convincing data that DFOG could induce the growth inhibition and arrest of cell cycle in G2/M phase in gastric cancer cells.

Effects of DFOG on FOXM1 expression in gastric cancer cells

The studies by Wang *et al*[18] have shown that FOXM1 signaling is over-expressed in pancreatic cancer and is involved in promotion of cell growth, and thus considered as a putative target for drug development. Therefore, we investigated whether DFOG could regulate FOXM1 signaling pathway. FOXM1 mRNA and protein expression in AGS cell line treated with DFOG and genistein for 24 h were decreased in a concentration-dependent manner (Figure 4A and B). We also found that FOXM1 protein expression was down-regulated by DFOG and genistein in SGC-7901 cells (Figure 4C).

Effects of DFOG on the expression of FOXM1 downstream target genes in gastric cancer cells

It is well known that FOXM1 has several downstream target genes, such as CDK1, Cdc25B, cyclin B, and $p27^{KIP1}$. We used Western blotting analysis to determine the expression of these genes, and found that DFOG and genistein inhibited the expression of CDK1, Cdc25B, cyclin B, and increased $p27^{KIP1}$ at the protein levels in AGS and SGC-701 cells (Figure 5A and B).

Effects of down-regulation of FOXM1 expression by siRNA on DFOG-induced cell growth inhibition in AGS cells

Down-regulation of FOXM1 by siRNA transfection showed less expression of FOXM1 protein in AGS cells, as confirmed by Western blot (Figure 6A).The down-regulation of FOXM1 expression significantly inhibited cell viability induced by DFOG (Figure 6B). DFOG plus FOXM1 siRNA inhibited cell growth to a greater degree compared with DFOG alone. FOXM1 siRNA transfection induces arrest of cell cycle in G2/M phase in AGS cells (Figure 6C). These results provide some molecular evidences suggesting that the DFOG-induced inhibition

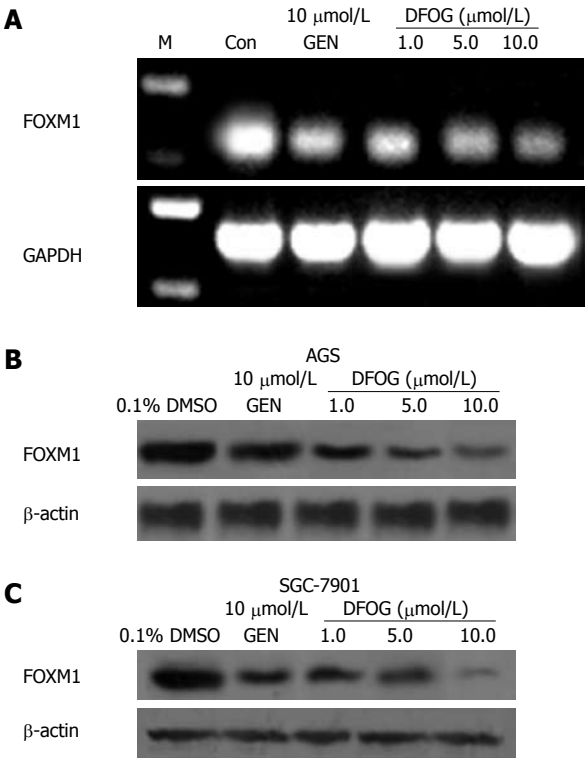


Figure 4 Down-regulation of forkhead box M1 expression by 7-difluoromethoxyl-5,4'-di-n-octylgenistein and genistein in AGS and SGC-7901. A: mRNA level using reverse transcription-polymerase chain reaction in AGS cell line; B: Protein level using Western blotting in AGS cell line; C: Decrease of forkhead box M1 (FOXM1) protein expression in SGC-7901 cell line. DMSO: Dimethyl sulfoxide; GEN: Genistein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

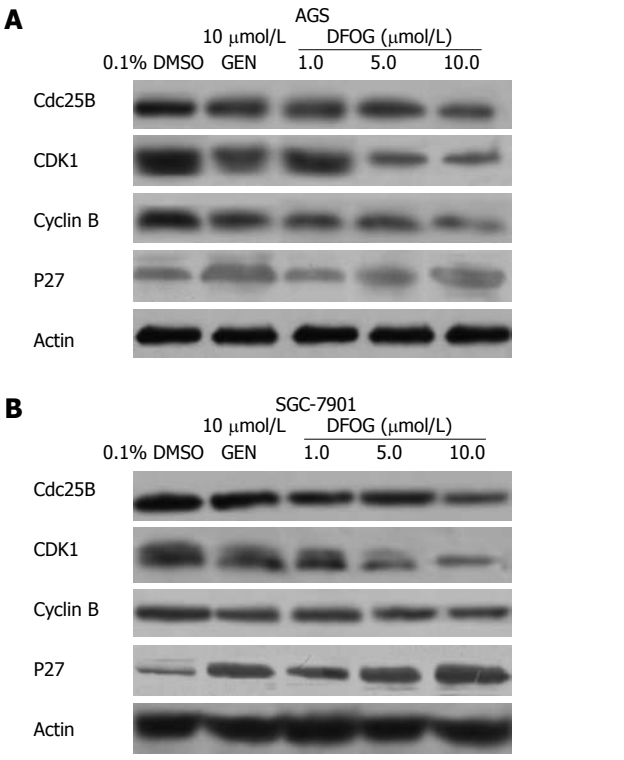


Figure 5 Modulation of the protein expressions of forkhead box M1 downstream target genes by 7-difluoromethoxyl-5,4'-di-n-octylgenistein and genistein. A: AGS cell line; B: SGC-7901 cell line. DMSO: Dimethyl sulfoxide; GEN: Genistein; DFOG: 7-difluoromethoxyl-5,4'-di-n-octylgenistein.

of the growth is mediated *via* inactivation of FOXM1 in gastric cancer cells.

Effects of over-expression of FOXM1 by cDNA transfection on DFOG-induced cell growth inhibition in AGS cells

Up-regulation of FOXM1 by cDNA transfection showed over-expression of FOXM1 protein in AGS cells, as confirmed by Western blotting (Figure 7A). The over-expression of FOXM1 rescued DFOG-induced cell viability inhibition to a certain degree (Figure 7B). These findings suggested that DFOG-inhibited cell growth is in part attributed to inactivation of FOXM1 signaling pathway in gastric cancer cells.

DISCUSSION

FOXM1 signaling has been demonstrated to maintain a balance between cell proliferation, differentiation and apoptosis, suggesting that abnormal activation of *FOXM1* gene is one of the characteristics of human cancers[22]. Increasing studies have shown over-expression of *FOXM1* gene in human cancer cells and tissues[14,23,24]. Thus, the development of agents targeting FOXM1 is likely to have a significant therapeutic impact on the treatment of human cancers, including gastric cancer. FOXM1 could be down-regulated by some drugs, such as antibiotic thiazole compound Siomycin A, thiostrepton, and epidermal growth factor receptor inhibitor gefitinib[25-27]. These observations clearly suggest that chemical compounds that target FOXM1 may act as anti-cancer drugs[27]. Wang *et al*[18] have shown that genistein may inhibit FOXM1 activation in pancreatic cancer cells, leading to apoptotic cell death. In the present study, we used two human gastric cancer cell lines, AGS and SGC-7901, which have high expression of FOXM1, and found that DFOG, a novel synthetic genistein analogue, and genistein could induce significant growth inhibition in the two cell lines, as evidenced by both MTT and clonogenic assay. Furthermore, DFOG and genistein inhibited the expression of FOXM1 and its target genes. Therefore, DFOG and genistein could mediate the cell growth inhibition partly *via* inactivation of FOXM1. Down-regulation of FOXM1 by siRNA together with DFOG treatment inhibited cell growth to a greater degree in AGS cells as compared with DFOG treatment alone. Up-regulation of FOXM1 by cDNA transfection showed over-expression of FoxM1 protein as confirmed by Western blotting analysis, and this over-expression in FOXM1 attenuated DFOG-induced cell growth inhibition in AGS cells. In view of these findings, we strongly believe that inactivation of FOXM1 by DFOG and genistein results in the down-regulation of its target genes, which are mechanistically linked with DFOG and genistein induced cell growth inhibition.

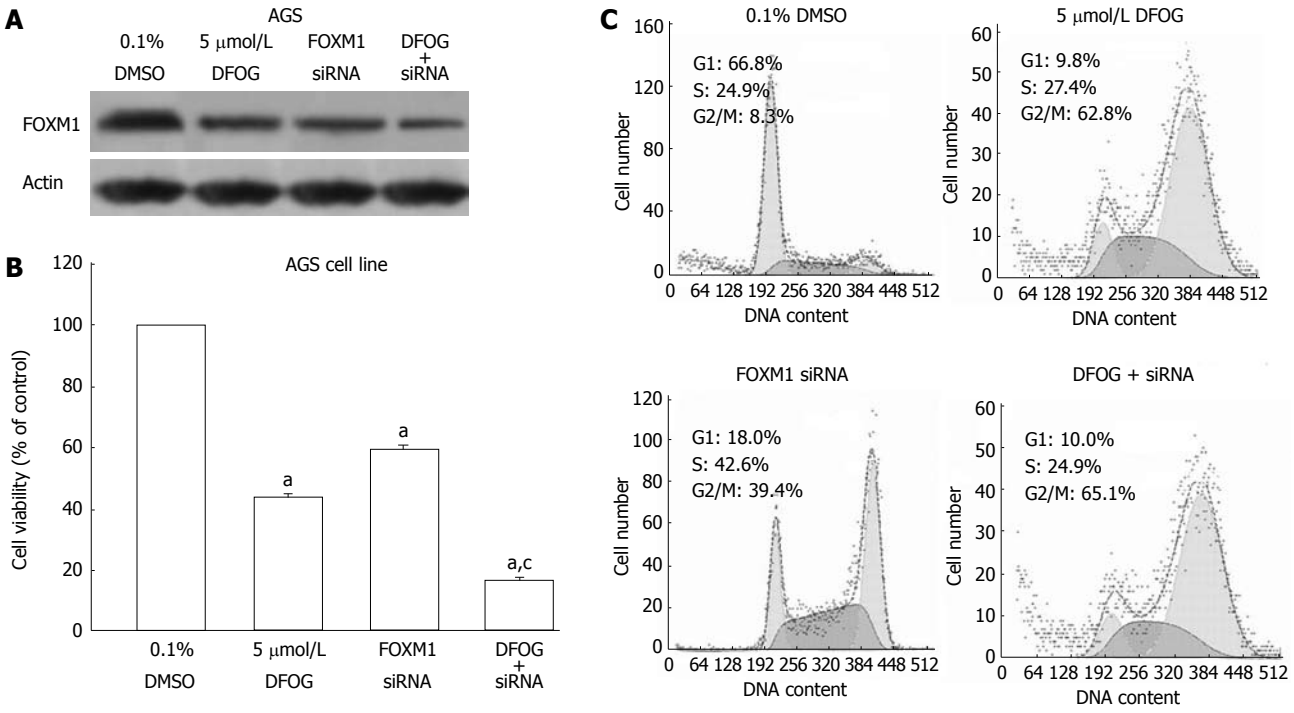


Figure 6 Forkhead box M1 small interfering RNA enhances the down-regulation of forkhead box M1 protein expression by 7-difluoromethoxyl-5,4'-di-n-octylgenistein and effects of forkhead box M1 small interfering RNA transfection or 7-difluoromethoxyl-5,4'-di-n-octylgenistein in AGS cell line. A: Western blotting analysis; B: The cell viability inhibitory effects of 7-difluoro methoxyl-5,4'-di-n-octylgenistein (DFOG) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide assay; C: Both on cell cycle distribution in AGS cells. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with 5 μmol/L DFOG or forkhead box M1 (FOXM1) small interfering RNA (siRNA) alone.

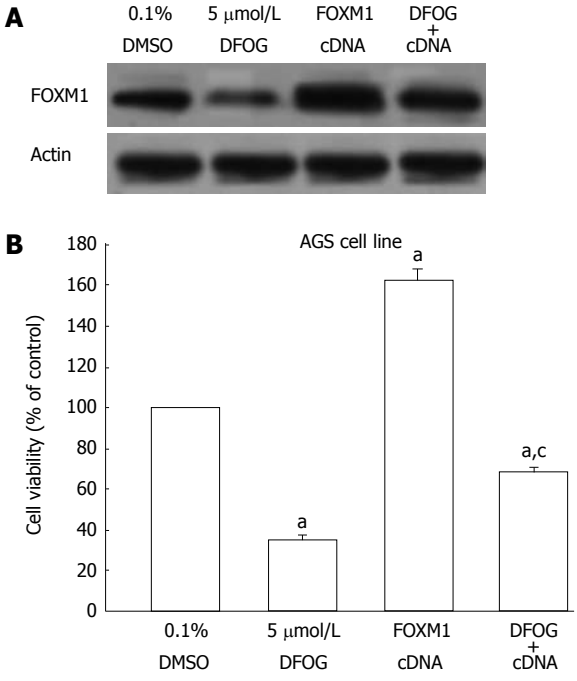


Figure 7 Forkhead box M1 cDNA transfection reduces the down-regulation of forkhead box M1 protein expression by 7-difluoromethoxyl-5,4'-di-n-octylgenistein in AGS cell line. A: Western blotting analysis; B: The cell viability inhibitory effects of 7-difluoro methoxyl-5,4'-di-n-octylgenistein (DFOG) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide assay. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with 5 μmol/L DFOG or forkhead box M1 (FOXM1) cDNA transfection alone.

Genistein has been found to induce G2 arrest and inhibit proliferation in a variety of cancer cell lines[28]. Because down-regulation of FOXM1 by DFOG reduced cell growth, we postulated that cell growth inhibition might result from cell cycle arrest in any specific phase of the cell cycle. We did find that FOXM1 down-regulation increased cell population in the G2/M phase and decreased cells in the G1/G0 and S phase. We also observed a marked reduction in cyclin B, Cdc25B and CDK1 expression in DFOG-treated cells. It is known that Cdc25B, cyclin B and CDK1 are the major effectors of the G2/M checkpoint response[29]. Diminished G1-S progression and growth rate was associated with increased expression of the *Cdk*I protein $p21^{CIP}$ and $p27^{KIP1}$, which have negative effects on cell cycle machinery by binding to various cyclin-Cdk complexes and inhibiting their activities[24,30]. In our study, the decreased cyclin B, cdc25B and Cdk1 and the increased expression of CdkI proteins, $p27^{KIP1}$, were strongly correlated with the altered cell cycle distribution phenotype and growth suppression. These results suggest that FOXM1 affects the gastric cancer cell cycle by regulating the expression levels of some cyclins (cyclin B), CDK (CDK1), CDK modulator (cdc25B) and CDK inhibitors ($p27^{KIP1}$).

In summary, we presented experimental evidence which strongly supports the role of DFOG, a novel synthetic genistein analogue, as an anti-tumor agent mediated through inactivation of FOXM1 signaling pathway.

However, further in-depth studies are needed to identify how DFOG could regulate the FOXM1 pathway, and to assess the anti-tumor activity mediated by the inactivation of FOXM1 either by genistein and DFOG or other synthetic compounds in pre-clinical animal models for the successful treatment of gastric cancer in the future. It is also tempting to speculate that the inactivation of FOXM1 together with the treatment of gastric tumor cells with conventional agents could be a useful strategy toward better treatment of human malignancies, especially gastric cancer.

COMMENTS

Background

Gastric cancer is one of the most common malignancies in the world and its incidence and mortality rank first in China. Recent data indicate that the mortality of gastric cancer in China tends to increase and it severely threatens the health and life of people. New therapeutic agents for this malignant disease are urgently needed.

Research frontiers

Genistein, 5,7,4'-trihydroxylisoflavone, a major component of soybean products, has been reported to possess anticancer activities. The authors synthesized a series of difluoromethoxylated genistein analogues and determined their protective effects against vascular endothelial cells. There have been few studies focusing on the anticancer effect of fluorinated genistein analogues.

Innovations and breakthroughs

The authors investigated whether the inhibitory effects of genistein and the novel synthetic genistein analogue 7-difluoromethoxyl-5, 4'-di-n-octylgenistein (DFOG) on the growth of gastric cancer cells could be attributed to modulation of Forkhead Box M1 (FOXM1) activity. It was found that DFOG and genistein down-regulated the FOXM1 expression and its downstream genes, including Cdc25B, CDK1, cyclin B and up-regulated $p27^{KIP1}$, resulting in the inhibition of gastric cancer cell growth.

Applications

These results provide strong evidences for the first time to support that FOXM1 is a rational target in gastric cancer, and the targeted inactivation of FOXM1, especially by genistein and its analogue DFOG, and provided new insight into strategies for better prevention of tumor progression and/or treatment of gastric cancer.

Terminology

Genistein, 5,7,4'-trihydroxylisoflavone, as one of the active constituents of soybean products, has been reported to possess anti-cancer activities. The DFOG is a novel synthetic genistein analogue.

Peer review

The novel anti-cancer drugs are mandatory to overcome cancer cells resistant to conventional anti-cancer drugs. In this paper the authors identified DFOG to be a good anti-cancer drug in gastric cancer cells. In addition, they also obtained the results to show FOXM1 as one of target molecules of DFOG. All data presented in this paper is acceptable.

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CASE REPORT

A case report of abdominal distention caused by herpes zoster

Su-Rong Zhou, Chuan-Yu Liu

Su-Rong Zhou, Department of Dermatology, The Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, Yantai 264000, Shandong Province, China
Chuan-Yu Liu, Department of Neurology, The Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, Yantai 264000, Shandong Province, China
Author contributions: Liu CY managed the case; Zhou SR wrote the paper.
Correspondence to: Chuan-Yu Liu, PhD, Department of Neurology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, No. 20 Yudong Road, Yantai 264000, Shandong Province, China. lcy32@126.com
Telephone: +86-535-6691999 Fax: +86-535-6240341
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Abstract

Gastrointestinal complications caused by herpes zoster are extremely rare. Here, we described a case of abdominal distention caused by herpes zoster. The patient was a 59-year-old female who suffered from unexplained paroxysmal and a burning pain on the right part of her waist and abdomen, accompanied by abdominal distention. Intestinal pseudo-obstruction was diagnosed by abdominal radiography. Distention of the right abdominal wall was still apparent after one month. In this report, we found that recovery from abdominal distention caused by herpes zoster is difficult and may require surgical intervention.

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Key words: Abdominal distention; Herpes zoster; Intestinal pseudo-obstruction; Ogilvie's syndrome

Peer reviewer: Dr. Shailendra Kapoor, University of Illinois, 7487 Sherwood Crossing Place No. 302, Mechanicsville, VA 23111, United States

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INTRODUCTION

Herpes zoster is a viral disease that typically manifests as a dermatological condition, marked by an irritating skin rash with blisters that is often limited to one side of the body; although rare, it has also been implicated in unusual gastrointestinal complications[1]. We treated a patient with abdominal distention caused by herpes zoster and report it below.

CASE REPORT

A 59-year-old female patient suffered from unexplained paroxysmal and a burning pain on the right part of her waist and abdomen for 2 wk. An erythra appeared on the affected area one week ago and the pain was exacerbated, interrupting the patient's sleep. A diagnosis of herpes zoster was made and the patient was administrated with valaciclovir (0.3 g *bid*), vitamin B_{12} (0.5 mg im *qd*), and ibuprofen and codeine phosphate sustained tablets (50 mg *bid*) for 3 wk. The patient had abdominal distention accompanied by dyspepsia, abdominal bloating and constipation, which showed no apparent changes after motilium treatment. Upon physical examination, laminal erythema was found on her left waist and abdomen accompanied by papulovesicles and small vesicles. The erythra showed zonal distribution not exceeding the midline. The abdomen was asymmetric with left distention, a lax abdominal wall and hyperalgesia. Two air-fluid levels appeared on the abdominal radiograph, which was diagnosed as intestinal pseudo-obstruction by surgical consultation. After treatment with fasting, fluid infusion,

enema and moxifloxacin (1 tablet *qd* × 3), the symptoms were relieved after 5 d. The diagnosis was modified as intestinal pseudo-obstruction caused by herpes zoster. Continued treatment included mecobalamin (1 tablet *tid*) to nourish the nerve and for heat-clearing and a detoxifying soft capsule (2 pills *tid*) to clear away heat and toxic materials and relax the bowels. After 15 d, the red spots disappeared, and the vesicles dried up, changed to scabs and dropped off, leaving anomalous pigmentation of the skin. At the same time, the pain was apparently alleviated, but the distention of the right abdominal wall and constipation persisted. On follow-up one month later, the distention of the right abdominal wall was still apparent, but the partial sense of pain decreased and the constipation was relieved.

DISCUSSION

Herpes zoster is a herpetic skin disease caused by varicella-zoster virus (VZV) infection, which can infect the surrounding sensory and motor nerves and their dominated skin areas. When the abdomen is involved, the infection can cause paralysis of the abdominal wall muscle[2], which may involve the autonomic nerve, the myenteron and small intestine smooth muscles, leading to the manifestation of intestinal pseudo-obstruction (Ogilvie's syndrome)[1]. The present patient was an overweight, middle-aged female. Due to two pregnancies, her abdomen became flabby. VZV infection in this area may cause nerve paralysis, a decrease in abdominal muscle tonus and abdominal distention. Simultaneously, indigestion, constipation and intestinal pseudo-obstruction appear due to the involvement of the autonomic nerves. Further consultation after one month showed no improvement of the abdominal wall distention. As recovery from this type of nerve paralysis is difficult, surgical intervention is sometimes necessary.

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Dr. Leif Percival Andersen, MD, Department of Infection Control 9101, Copenhagen University Hospital, Rigshospitalet, Juliane Maries Vej 18, DK-2100 Copenhagen, Denmark

Ekaterini Chatzaki, PhD, Assistant Professor, Laboratory of Pharmacology, Faculty of Medicine, Dragana, Alexandroupolis, 68100 Thrace, Greece

Edward J Ciaccio, PhD, Research Scientist, Department of Medicine, HP 804, Columbia University, 180 Fort Washington Avenue, New York, NY 10032, United States

Andrew S Day, MB, ChB, MD, FRACP, AGAF, Associate Professor, Department of Paediatrics, University of Otago, Christchurch, P.O. Box 4345, Christchurch 8140, New Zealand

Giuliana Decorti, MD, PhD, Department of Life Sciences, University of Trieste, Via L. Giorgieri n° 7, 34127 Trieste, Italy

Magdy El-Salhy, MD, PhD, Professor, Chief Gastroenterologist, Section for Gastroenterology, Department of Medicine, Stord Helse-Fonna Hospital, Box 4000, 54 09 Stord, Norway

Francesco Franceschi, MD, PhD, Assistant Professor, Internal Medicine, Catholic University of Rome, Gemelli Hospital, Largo A. Gemelli, 8, 00168 Rome, Italy

Oliver Grundmann, PhD, Clinical Assistant Professor, Department of Medicinal Chemistry, College of Pharmacy, University of Florida, 1600 SW Archer RD, Room P6-20, Gainesville, FL 32610-0484, United States

Masahiro Iizuka, MD, PhD, Director, Akita Health Care Center, Akita Red Cross Hospital, 3-4-23, Nakadori, Akita 010-0001, Japan

Eric R Kallwitz, MD, Assistant Professor, Department of Medicine, University of Illinois, 840 S Wood Street, 7th Floor, MC 787, Chicago, IL 60612, United States

Hyo-Cheol Kim, MD, Clinical Assistant Professor in Vascular Intervention Section, Department of Radiology, Seoul National University Hospital, 28 Yongon-dong, Chongno-gu, Seoul 110-744, South Korea

Young Chul Kim, PhD, Professor of Toxicology, Seoul National University, College of Pharmacy, San 56-1 Shinrim-Dong, Kwanak-Ku, Seoul 151-742, South Korea

Javier San Martín, Chief, Gastroenterology and Endoscopy, Sanatorio Cantegril, Av. Roosevelt y P 13, 20100 Punta del Este, Uruguay

B Mittal, PhD, Professor, Department of Genetics, Sanjay Gandhi Medical Institute, Lucknow 226014, India

Hikaru Nagahara, MD, PhD, Professor, Aoyama Hospital, Tokyo Women's Medical University, 2-7-13 Kita-Aoyama, Minatoku, Tokyo 107-0061, Japan

Atsushi Nakajima, Professor, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

Noriko Nakajima, MD, PhD, Associate Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Nihon University School of Medicine, 1-8-13 Kandasurugadai Chiyoda-ku, Tokyo 101-8309, Japan

Samiran Nundy, MD, Department of Surgical Gastroenterology and Liver Transplantation, Sir Ganga Ram Hospital, New Delhi 110060, India

Bronislaw L Slomiany, PhD, Professor, Research Center, C-875, UMDNJ-NJ Dental School, 110 Bergen Street, PO Box 1709, Newark, NJ 07103-2400, United States

Branko Stefanovic, PhD, Department of Biomedical Sciences, College of Medicine, Florida State University, 1115 W.Call st, Tallahassee, FL 32306-4300, United States

Hans L Tillmann, Professor, Medizinische Klinik und Poliklinik II, University Leipzig, Philipp Rosenthal, Str. 27, 04103 Leipzig, Germany

Kentaro Yoshika, Associate Professor, Division of Gastroenterology, Department of I, Fujita Health University School of Medicine, 1-98 Dengakugakubo, Kutsukade, Toyoake 470-1190, Japan

Katsutoshi Yoshizato, PhD, Academic Advisor, Phoenix Bio Co., Ltd., 3-4-1 Kagamiyama, Higashihiroshima 739-0046, Japan

Angelo Zullo, MD, Gastroenterologia ed Endoscopia Digestiva, Ospedale Nuovo Regina Margherita, Via E. Morosini, 30, 00153 Roma, Italy

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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GENERAL INFORMATION

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The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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Editor-in-chief
Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University of Pisa, Director of General Medicine 2 Unit University Hospital of Pisa, Via Roma 67, 56124 Pisa, Italy

Myung-Hwan Kim, MD, PhD, Professor, Head, Department of Gastroenterology, Director, Center for Biliary Diseases, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-2dong, Songpa-gu, Seoul 138-736, South Korea

Kjell Öberg, MD, PhD, Professor, Department of Endocrine Oncology, Uppsala University Hospital, SE-751 85 Uppsala, Sweden

Matt D Rutter, MBBS, MD, FRCP, Consultant Gastroenterologist, Senior Lecturer, Director, Tees Bowel Cancer Screening Centre, University Hospital of North Tees, Durham University, Stockton-on-Tees, Cleveland TS19 8PE, United Kingdom

Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, CA, 5901 E. Seventh Str., Long Beach, CA 90822, United States

Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

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In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

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AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no less than 140 words); RESULTS (no less than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Room 903, Building D, Ocean International Center,
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Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, CA, 5901 E. Seventh Str., Long Beach, CA 90822, United States

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Jian-Xia Cheng, Director
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
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EDITORIAL

MicroRNAs in inflammatory bowel disease - pathogenesis, diagnostics and therapeutics

Mehmet Coskun, Jacob Tveiten Bjerrum, Jakob Benedict Seidelin, Ole Haagen Nielsen

Mehmet Coskun, Jacob Tveiten Bjerrum, Jakob Benedict Seidelin, Ole Haagen Nielsen, Department of Gastroenterology, Medical Section 54 O3, University of Copenhagen, Herlev Hospital, DK-2730 Herlev, Denmark
Mehmet Coskun, Jacob Tveiten Bjerrum, Department of Cellular and Molecular Medicine, the Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark
Jakob Benedict Seidelin, Department of Internal Medicine I, University of Copenhagen, Bispebjerg Hospital, DK-2400 Copenhagen NV, Denmark
Author contributions: Coskun M analyzed the literature, wrote the manuscript and the final revision of the article; Bjerrum JT provided intellectual input, advice, and contributed to the writing and final revision of the manuscript; Seidelin JB and Nielsen OH contributed to the conceptual design, drafting of the manuscript and revising it critically for important intellectual content; and all authors approved the final submitted manuscript.
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Correspondence to: Mehmet Coskun, PhD, Department of Gastroenterology, Medical Section 54 O3, University of Copenhagen, Herlev Hospital, Herlev Ringvej 75, DK-2730 Herlev, Denmark. mehmet.coskun@regionh.dk
Telephone: +45-38-683421 Fax: +45-38-684009
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Abstract

The pathogenesis of inflammatory bowel disease (IBD) is complex and largely unknown. Until recently, research has focused on the study of protein regulators in inflammation to reveal the cellular and molecular networks in the pathogenesis of IBD. However, in the last few years, new and promising insights have been generated from studies describing an association between an altered expression of a specific class of non-coding RNAs, called microRNAs (miRs or miRNAs) and IBD. The short (approximately 22 nucleotides), endogenous, single-stranded RNAs are evolutionary conserved in animals and plants, and regulate specific target mRNAs at the post-transcriptional level. MiRNAs are involved in several biological processes, including development, cell differentiation, proliferation and apoptosis. Furthermore, it is estimated that miRNAs may be responsible for regulating the expression of nearly one-third of the genes in the human genome. Thus, miRNA deregulation often results in an impaired cellular function, and a disturbance of downstream gene regulation and signaling cascades, suggesting their implication in disease etiology. Despite the identification of more than 1900 mature human miRNAs, very little is known about their biological functions and functional targets. Recent studies have identified dysregulated miRNAs in tissue samples of IBD patients and have demonstrated similar differences in circulating miRNAs in the serum of IBD patients. Thus, there is great promise that miRNAs will aid in the early diagnosis of IBD, and in the development of personalized therapies. Here, we provide a short review of the current state-of-the-art of miRNAs in IBD pathogenesis, diagnostics and therapeutics.

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Key words: Biomarker; Crohn's disease; Diagnostics; Inflammatory bowel disease; MicroRNA; Therapeutics; Ulcerative colitis

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INTRODUCTION

Inflammatory bowel disease (IBD), whose incidence is

increasing[1], comprises a number of intestinal chronic relapsing inflammatory disorders[2-4], among which ulcerative colitis (UC)[5] and Crohn's disease (CD)[6] are the two main entities. The pathogenesis of IBD remains largely unknown[7,8], but involves a complex interaction between genetic, environmental, and immunological factors[9,10]. In this context, research on microRNAs (miRs or miRNAs) is a promising new research, providing novel insights into the pathogenesis of IBD, biomarker identification, and treatment. Since their discovery and initial characterization in 1993[11,12], the number of miRNA sequences deposited in the miRBase database (Sanger Database) has grown continuously[13]. Numerous investigations have uncovered the key roles of miRNAs in several physiological networks[14-18]. Given their ability to target mRNAs and their predicted regulation of the expression of nearly one-third of all human transcripts, miRNAs have been linked to critical processes underlying development and tissue homoeostasis. Thus, dysregulation of miRNAs is implicated in the pathogenesis of several human diseases[18-26], including gastrointestinal disorders, such as cancer and inflammation[27-29]. Actually, loss of intestinal miRNAs in mouse models has been shown to impair differentiation of intestinal cells and epithelial barrier function, resulting in inflammation[30].

The discovery and role of miRNAs in IBD, particularly their role in cell signaling, seems to offer a new way of understanding this chronic disease and gives rise to new diagnostic tools and potential therapeutic strategies. Thus, it is possible that some miRNAs could serve as biomarkers for IBD, aiding early diagnosis, and lead to the development of personalized therapies.

Here we provide a short review of the current research in miRNAs in IBD; including pathogenesis, diagnostics and therapeutics.

MiRNA BIOGENESIS

MiRNAs are a recently discovered class of short (18-24 nucleotides in length), endogenous, non-coding single-stranded RNAs that regulate gene expression by controlling the stability and translation of protein-coding mRNAs[26,31,32]. A number of biological processes are regulated by miRNAs, including cell differentiation, proliferation, apoptosis, and cell cycle control[30,33,34]. Long primary miRNA transcripts (pri-miRNA) are initially transcribed by RNA polymerase Ⅱ or Ⅲ in the nucleus, and are then cleaved to precursor hairpin (pre-miRNA) by the microprocessor complex, Drosha and DiGeorge syndrome critical region gene 8[35-37]. Next, the pre-miRNAs are exported into the cytoplasm and further cleaved to mature miRNAs by the RNase Ⅲ endonuclease, Dicer, in a complex with a trans-activator RNA binding protein[38-40]. One RNA strand from the mature miRNA is incorporated into the RNA-induced silencing complex, and guides this complex to the 3' untranslated regions of "target" mRNA sequences, which in mammalian cells induces target mRNA degradation and suppresses protein expression[31,32,41-44].

ABBERANT EXPRESSION OF MiRNAS IN INFLAMMATORY BOWEL DISEASE

In 2008, Wu *et al*[45] were the first to report miRNA expression in colonic mucosa samples from IBD patients. They identified 11 miRNAs that were differentially expressed in active UC *vs* controls (Table 1)[45] and demonstrated an inverse relationship between macrophage inflammatory peptide-2α (previously shown to be implicated in IBD[46]) and miR-192. Similarly, Bian *et al*[47] found miR-150 to be significantly upregulated in inflamed colonic mucosa of UC patients, as compared to controls (Table 1), and they established an inverse correlation between miR-150 and its target, *c-Myb*[48], a proto-oncogene that is involved in apoptosis. Consequently, these two studies have exposed new and important insights into the pathogenesis of IBD.

In 2010, three studies[49-51] identified altered miRNA expression in IBD tissue but without concomitant functional analyses. In a cohort of 12 controls and 12 active UC patients, Takagi *et al*[49] reported miR-21 and miR-155 to be significantly upregulated in inflamed colonic UC tissue (Table 1). In a second study, Wu *et al*[50] assayed the expression of 467 miRNAs in patients with sigmoid CD and in patients with active terminal ileal CD. They found five miRNAs to be associated with active sigmoid CD, and four miRNAs were significantly increased in active ileal CD, as compared to control tissues (Table 1)[50]. These reports were followed by a similar study by Fasseu *et al*[51] evaluating the expression of more than 300 miRNAs in colonic tissue samples of UC and CD patients using quantitative real-time polymerase chain reaction analysis. Several miRNAs were differentially expressed in accordance with disease type, but with a large number in common to both groups (Table 1). They identified a set of eight miRNAs (miR-26a, miR-29a, miR-29b, miR-30c, miR-126*, miR-127-3p, miR-196a, and miR-324-3p) defining quiescent IBD *vs* controls, and a distinct subset of 15 miRNAs that could differentiate between quiescent UC and CD (n = 16) (Table 1)[51]. These three studies illustrate the potential use of miRNAs as biomarkers and the possibilities of developing miRNA profile-based diagnostic tools.

Other recent studies have focused on certain specific miRNAs and their association with target genes. Hence, Pekow *et al*[52] reported an inverse correlation of the tumor suppressors miR-143 and miR-145 with their target genes, *IRS-1* (miR-145), and *K-RAS*, *API-5* and *MEK-2* (miR-143). Similarly, Nguyen *et al*[53] demonstrated decreased levels of miR-7 in inflamed CD colonic tissue, where expression of its target, CD98, was upregulated compared with control tissue. Dysregulation of CD98 interferes with the natural proliferation and differentiation of enterocytes. These two studies[52,53] not only provided new information on the pathophysiology of the well known, but poorly understood, inflammation-driven neoplastic progress in the colonic mucosa of UC, but also provided targets for future therapeutic interventions.

Some studies have focused on single miRNAs and their relation to single-nucleotide polymorphisms (SNPs). Brest *et al*[54] found increased expression of miR-196 re-

Table 1 Overview of dysregulated miRNAs reported to date in tissues from inflammatory bowel disease patients

miRNAs	Sample type	Population (*n*)	Approach	Reference
miRs-192, 375, 422b, **16, 21, 23a, 24, 29a, 126, 195,** and **let-7f**	Sigmoid colon biopsies	Active UC (*n* = 15) *vs* healthy controls (*n* = 15)	Microarray and qRT-PCR	Wu *et al*[45]
miR-**21** and miR-**155**	Sigmoid colon biopsies	Active UC (*n* = 12) *vs* healthy controls (*n* = 12)	Microarray and qRT-PCR	Takagi *et al*[49]
miRs-19b, 629, **23b, 106a,** and **191**	Sigmoid colon biopsies	Active CD (*n* = 5) *vs* healthy controls (*n* = 13)	Microarray and qRT-PCR	Wu *et al*[50]
miRs-**16, 21, 223,** and **594**	Terminal ileum biopsies	Active CD (*n* = 6) *vs* healthy controls (*n* = 13)	Microarray and qRT-PCR	Wu *et al*[50]
miRs-188-5p, 215, 320a, 346, **7, 31, 135b, 223, 29a, 29b, 126*, 127-3p**, and **324-3p**	Colon biopsies	Active UC (*n* = 8) *vs* healthy controls (*n* = 8)	qRT-PCR	Fasseu *et al*[51]
miRs-188-5p, 215, 320a, 346, **196a, 29a, 29b, 126*, 127-3p**, and **324-3p**	Colon biopsies	Inactive UC (*n* = 8) *vs* healthy controls (*n* = 8)	qRT-PCR	Fasseu *et al*[51]
miRs-**9, 126, 130a, 181c, 375, 26a, 29b, 30b, 34c-5p, 126*, 127-3p, 133b, 155, 196a, 324-3p, 21, 22, 29c, 31, 106a, 146a, 146b-3p**, and **150**	Colon biopsies	Active CD (*n* = 8) *vs* healthy controls (*n* = 8)	qRT-PCR	Fasseu *et al*[51]
miRs-**9*, 30a*, 30c, 223, 26a, 29b, 30b, 34c-5p, 126*, 127-3p, 133b, 155, 196a, 324-3p, 21, 22, 29c, 31, 106a, 146a, 146b-3p,** and **150**	Colon biopsies	Inactive CD (*n* = 8) *vs* healthy controls (*n* = 8)	qRT-PCR	Fasseu *et al*[51]
miRs-150, 196b, 199a-3p, 199b-5p, 223, and 320a	Colon biopsies	Inactive UC (*n* = 8) *vs* Inactive CD (*n* = 8)	qRT-PCR	Fasseu *et al*[51]
miR-7	Colon biopsies	Active CD (*n* = 8) *vs* healthy controls (*n* = 6)	qRT-PCR	Nguyen *et al*[53]
miR-**150**	Colon biopsies	Active UC (*n* = 5) *vs* healthy controls (*n* = 4)	qRT-PCR	Bian *et al*[47]
miR-**196**	Colon biopsies	Active CD (*n* = 83) *vs* healthy controls (*n* = 67)	qRT-PCR and ISH	Brest *et al*[54]
miR-143 and miR-145	Colon biopsies	Active UC (*n* = 8) *vs* healthy controls (*n* = 8)	qRT-PCR	Pekow *et al*[52]

Both downregulated miRNAs (miRs) and upregulated (bold characters) are shown. CD: Crohn's disease; ISH: *In situ* hybridization; UC: Ulcerative colitis; qRT-PCR: Quantitative real-time polymerase chain reaction.

stricted to intestinal epithelial cells within inflamed CD as compared to controls. They showed that miR-196 binds to, and correlates with, a decreased expression of the immunity-related GTPase M (*IRGM*) protective variant (c.313C) during inflammatory conditions, but not with the *IRGM* c.313C>T polymorphism, $IRGM^T$, which is strongly associated with CD in European populations[55]. In a similar study, Zwiers *et al*[56] found that the mutation (rs10889677 C>A) in the *IL-23R* gene, associated with IBD, results in loss of its binding sites for let-7e and let-7f miRNAs, leading to sustained *IL-23R* expression *in vitro*, which contribute to the chronicity of IBD. Thus, these studies indicate that single SNPs located at miRNA-binding sites are likely to affect the expression of their targets and that they might contribute to the pathogenesis of IBD.

In a semi-invasive approach and with diagnostic intentions, few studies have used whole blood instead of colonic tissue. As a proof of concept, Wu *et al*[57] performed a microarray-based study on whole blood from IBD patients and found a panel of differentially expressed miRNAs (Table 2) that enabled them to distinguish active IBD subtypes from each other and from controls. In a similar study by Zahm *et al*[58], higher concentrations of 11 miRNAs (Table 2) were found in the sera of pediatric CD patients *vs* controls. Receiver operating characteristic analyses resulted in a diagnostic sensitivity above 80% for CD[58]. Recently, Paraskevi *et al*[59] identified that 11 circulating miRNAs that were differentially expressed in blood samples from active CD *vs* controls, and identified a set of six miRNAs that were significantly elevated in active UC *vs* healthy controls (Table 2). Similarly, Duttagupta *et al*[60] found seven circulating miRNAs that were differentially expressed in UC patients *vs* controls (Table 2).

Although these studies are preliminary, they demonstrate the possibilities of developing a much-needed semi-invasive test based on differentially expressed peripheral blood miRNAs.

FUTURE USE IN DISEASE DIAGNOSTICS AND THERAPEUTICS

The studies described above have significantly increased our knowledge regarding the pathogenesis of IBD and have demonstrated the usefulness of miRNAs, not only as potential biomarkers, but also as latent targets for therapeutic interventions. However, a range of obvious challenges lie ahead, especially as identification of new miRNAs is an ongoing process. In 2008, Wu *et al*[45] used a miRNA microarray containing 553 known human miRNA genes, but currently more than 1900 human miRNA sequences are known[13], and new ones are identified on an almost daily basis. Consequently, this area of research is in its infancy, and future studies in the field of IBD need to focus on miRNA sequencing and using

Table 2 Overview of dysregulated circulating miRNAs reported to date in sera of inflammatory bowel disease patients

miRNAs	Sample type	Population (*n*)	Approach	Reference
miRs-149*, miRplus-F1065, **199a-5p, 362-3p, 340*, 532-3p**, and **miRplus-E1271**	Peripheral blood	Active CD (*n* = 14) *vs* healthy controls (*n* = 13)	Microarray and qRT-PCR	Wu *et al*[57]
miR-149* and miR-**340***	Peripheral blood	Inactive CD (*n* = 5) *vs* healthy controls (*n* = 13)	Microarray and qRT-PCR	Wu *et al*[57]
miRs-505*, **28-5p, 151-5p, 103-2*, 199a-5p, 340*, 362-3p, 532-3p**, and **miRplus-E1271**	Peripheral blood	Active UC (*n* = 13) *vs* healthy controls (*n* = 13)	Microarray and qRT-PCR	Wu *et al*[57]
miRs-505*, **28-5p, 103-2*, 149*, 151-5p, 340*, 532-3p**, and **miRplus-E1153**	Peripheral blood	Active UC (*n* = 10) *vs* active CD (*n* = 14)	Microarray and qRT-PCR	Wu *et al*[57]
miRs-**195, 16, 93, 140, 30e, 20a, 106a, 192, 21, 484**, and **let-7b**	Serum	Active CD (*n* = 46) *vs* healthy controls (*n* = 32)	LDA qRT-PCR	Zahm *et al*[58]
miRs-**16, 23a, 29a, 106a, 107, 126, 191, 199a-5p, 200c, 362-3p**, and **532-3p**	Peripheral blood	Active CD (*n* = 128) *vs* healthy controls (*n* = 162)	qRT-PCR	Paraskevi *et al*[59]
miRs-**16, 21, 28-5p, 151-5p, 155**, and **199a-5p**	Peripheral blood	Active UC (*n* = 88) *vs* healthy controls (*n* = 162)	qRT-PCR	Paraskevi *et al*[59]
miRs-**188-5p, 422a, 378, 500, 501-5p, 769-5p**, and **874**	Peripheral blood	UC (*n* = 20) *vs* healthy controls (*n* = 20)	qRT-PCR	Duttagupta *et al*[60]

Downregulated miRNAs (miRs) as well as upregulated (bold characters) are shown. CD: Crohn's disease; LDA: Low-density array; UC: Ulcerative colitis; qRT-PCR: Quantitative real-time polymerase chain reaction.

larger cohorts to address two important challenges: (1) identification of all of the miRNAs that are consistently dysregulated in IBD; and (2) to identify all of the targets of the miRNAs involved in IBD. If these challenges are met, the subsequent possibilities regarding diagnostics and therapeutics seem endless.

The potential clinical use of miRNAs is best illustrated by the most investigated and well-described miRNA, miR-21, also classified as an "oncomiR"[61,62]. The almost omnipresent overexpression of miR-21 in human cancers[62-64], including colorectal cancer, provides new options for cancer therapy[65]. Interestingly, as listed in Tables 1 and 2, miR-21 is the only miRNA commonly found to be upregulated in inflamed tissue or sera of IBD patients[45,49-51,58]. Thus, miR-21 is a potential biomarker for active IBD. It may also be of significant importance for the pathogenesis of IBD, as expression of miR-21 is mediated by nuclear factor-κB[66] - a key transcription factor involved in the pathogenesis of various human diseases, including IBD[67,68]. Unfortunately, the importance of these miRNA pathways in disease pathogenesis is unknown. Thus, functional studies are needed to address the role of miRNAs in IBD and particularly their role in cell signaling.

CONCLUSION

Currently, several clinical trials are testing the therapeutic efficacy of miRNA-based therapies; one such miRNA-target drug is "miravirsen"[69], a specific inhibitor of miR-122 that is currently in phase II clinical trials for Hepatitis C infections[70]. Within the next several years, further studies will undoubtedly provide a basis for more successful clinical trials and provide more insights in to the efficacy of miRNA-based therapeutics, including IBD.

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FRONTIER

Post-endoscopic retrograde cholangio-pancreatography pancreatitis: Is time for a new preventive approach?

Stella Tammaro, Roberta Caruso, Francesco Pallone, Giovanni Monteleone

Stella Tammaro, Division of Gastroenterology, Azienda Ospedaliera Universitaria, 84131 Salerno, Italy
Roberta Caruso, Francesco Pallone, Giovanni Monteleone, Department of Systems Medicine, Division of Gastroenterology, University of Rome Tor Vergata, 00133 Rome, Italy
Author contributions: Tammaro S collected the materials and contributed to writing the manuscript; Caruso R and Monteleone G discussed the topic and contributed to writing the paper; Pallone F supervised the manuscript.
Supported by The Fondazione "Umberto Di Mario" Onlus, Rome
Correspondence to: Giovanni Monteleone, MD, PhD, Department of Systems Medicine, Division of Gastroenterology, University of Rome Tor Vergata, 00133 Rome, Italy. gi.monteleone@med.uniroma2.it
Telephone: +39-6-72596158 Fax: +39-6-72596391
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Abstract

Acute pancreatitis is the most common serious complication of endoscopic retrograde cholangio-pancreatography (ERCP) and its incidence may exceed 25% in some high-risk patient subsets. In some patients, pancreatitis may follow a severe course with pancreatic necrosis, multiorgan failure, permanent disability and even death. Hence, approaches which minimize both the incidence and severity of post-ERCP pancreatitis are worth pursuing. Pancreatic stents have been used with some success in the prevention of post-ERCP, while so far pharmacological trials have yielded disappointing results. A recent multicenter, randomized, placebo-controlled, double-blind trial has shown that rectally administered indomethacin is effective in reducing the incidence of post-ERCP pancreatitis, the occurrence of episodes of moderate-to-severe pancreatitis and the length of hospital stay in high-risk patients. These results together with the demonstration that rectal administration of indomethacin is not associated with enhanced risk of bleeding strongly support the use of this drug in the prophylaxis of post-ERCP pancreatitis.

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Key words: Endoscopic retrograde cholangiopancreatography; Post-endoscopic retrograde cholangiopancreatography pancreatitis; Non-steroidal anti-inflammatory drugs; Indomethacin; Pancreatitis prevention

Peer reviewers: Sara Regnér, MD, PhD, Department of Surgery, Institution of Clinical Sciences Malmo, Malmo University Hospital, Ing 82B, Universitetssjukhuset MAS, SE-20502 Malmö, Sweden; Atsushi Nakajima, Professor, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan; Eyvind J Paulssen, MD, PhD, Department of Gastroenterology, University Hospital of North Norway, PO Box 83, N-9038 Tromsø, Norway

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INTRODUCTION

Physicians sometimes make a strenuous effort to cope with illnesses which develop de novo following diagnostic and/or therapeutic interventions. One example of such a struggle is the management of complications occurring in patients undergoing endoscopic retrograde cholangiopancreatography (ERCP), a procedure used to both diagnose and treat diseases of the pancreaticobiliary tree[1-4]. Indeed despite recent advances in technology and experience of gastroenterologists, the use of ERCP can be accompanied by perforation, bleeding and pancreatitis[5,6]. Pancreatitis is the most common complication of

ERCP[5,7-10]; its frequency varies between 1% and 10% in average risk patients[6,7,11-13] but can exceed 20% in high-risk patient subset[14-16].

DIAGNOSIS, SEVERITY AND RISK FACTORS OF POST-ERCP PANCREATITIS

The diagnosis of post-ERCP pancreatitis is based on the presence of pancreatic-type abdominal pain, increase in serum amylase at least 3 times above the upper limit of normal 24 h after the procedure and need of hospitalization or prolongation of a planned hospital admission[7]. Although various criteria have been used to define the severity of pancreatitis[17-21], the length of hospitalization is now considered a major factor in the evaluation of disease severity. Mild pancreatitis is defined as need for an unplanned hospital stay or an extension of a planned hospital stay by 2-3 d; moderate pancreatitis requires 4-10 d of hospitalization and severe pancreatitis results in a hospitalization of > 10 d. Additionally, pancreatitis can be defined severe if it leads to the development of pancreatic necrosis or pseudocyst, or requires percutaneous or surgical intervention[7]. While most patients develop a mild pancreatitis with rapid and full recovery, a small percentage may follow a severe course with pancreatic necrosis, multiorgan failure, permanent disability, and even death[7,11,12,22]. Hence, approaches which minimize both the incidence and severity of post-ERCP pancreatitis are worth pursuing. Some patient-related (i.e., known or suspected dysfunction of sphincter of Oddi, female gender and previous pancreatitis) and procedure-related (i.e., precut sphincterotomy, pancreatic injection, ampullectomy) characteristics are definite independent risk factors for post-ERCP pancreatitis and may be useful for the stratification of patients into low-risk or high-risk categories[23]. Further factors which may influence the outcome of ERCP and the risk of post-ERCP pancreatitis include availability of equipment and adequacy of endoscopic, radiologic and nursing support[23].

PATHOGENESIS AND PREVENTION OF POST-ERCP PANCREATITIS

Various types of injury (i.e., mechanical injury to the papilla from cannulation, chemical injury due to ionic contrast agents, hydrostatic injury as a result of excessive contrast injection and thermal injury from electrocautery), developing during the procedure, can impair the drainage from the pancreas and favor activation of factors that cause tissue damage[24,25]. Although the exact mechanism driving post-ERCP pancreatitis is not fully understood, circumstantial evidence suggests that, once activated, the pathways of inflammation promote intraluminal activation of proteolytic enzymes, autodigestion of the organ, impaired acinar secretion, and then massive synthesis of chemokines and proinflammatory cytokines, which amplify the ongoing inflammatory process[25-29]. Strategies that interrupt one or more of these critical steps could help prevent post-ERCP pancreatitis. There is a wide consensus among gastroenterologists that the ideal pharmacological agent for preventing post-ERCP pancreatitis should have a short administration time, be well tolerated with a low side-effect profile and cost-effective.

The anti-inflammatory activity of indomethacin was first described in 1963, and the drug has been used to treat several rheumatological conditions including osteo- and rheumatoid arthritis[30,31]. Indomethacin shares important pharmacological properties with other non-steroidal anti-inflammatory drugs (NSAIDs), particularly aspirin, and inhibits cyclooxygenase, phospholipase A2 and neutrophil-endothelial interactions, which are supposed to play a major role in the pathogenesis of pancreatitis[32-35]. Previous reports have shown that NSAIDs, including indomethacin, reduce experimental pancreatitis-driven lethality in rodents[36] and rectal indomethacin decreases the number of d with pain and number of opiate injections in patients with acute pancreatitis[37]. Moreover, three recent meta-analyses[38-40] of four prospective randomized placebo-controlled trials showed that rectal administration of 100 mg diclofenac immediately after the procedure or 100 mg indomethacin immediately before the procedure was effective in reducing pancreatitis, with a pooled relative risk after administration of 0.36 (95% CI: 0.22-0.60)[15,41-43]. Further analysis showed that prevention of post-ECRP pancreatitis by NSAIDs occurred in patients with either low-risk or high-risk. In particular, Murray *et al*[42] showed that 100 mg of rectal diclofenac administered to high-risk patients (i.e., patients undergoing pancreatography or with manometrically documented sphincter of Oddi dysfunction) upon arrival in the recovery area significantly reduced the incidence of post-ERCP pancreatitis [7/110 (6.4%) *vs* 17/110 (15.5%) in the placebo group]. Similarly, Khoshbaten *et al*[43] showed that 100 mg of rectal diclofenac administered to high-risk patients upon arrival in the recovery area was superior to placebo in reducing the incidence of post-ERCP pancreatitis [2/50 (4%) *vs* 13/50 (26%) in the placebo group]. Along the same line is the paper published by Elmunzer *et al*[44] in a recent issue of *The New England Journal of Medicine* and showing that rectally administered indomethacin significantly reduces the incidence of post-ERCP pancreatitis in high-risk patients. The authors performed a multicenter, randomized, placebo-controlled, double-blind trial in which 602 patients were assigned to receive a single dose of rectal indomethacin (100 mg) or placebo immediately after ERCP[44]. The selected patients showed an increased baseline risk of post-ERCP pancreatitis on the basis of patient- and procedure-related independent risk factors (i.e., sphincter of Oddi dysfunction, a history of post-ERCP pancreatitis, pancreatic sphincterotomy and ampullectomy). Overall 79/602 (13.1%) patients developed post-ERCP pancreatitis and this complication was more frequent in patients receiving placebo [52/307 (16.9%)] than in those treated with indomethacin [27/295 (9.2%)]. Moreover, indomethacin reduced the occurrence of episodes of moderate-to-severe pancreatitis [13 (4.4%)

vs 27 (8.8%) in the placebo group]. Consequently, the median length of hospital stay was 0.5 d shorter in the group of patients treated with indomethacin[44]. Interestingly, in accordance with previous studies evaluating NSAIDs in the prevention of post-ERCP pancreatitis[6,45], the benefit of rectal administration of indomethacin was not associated with enhanced risk of bleeding, as this event was documented in 4 patients receiving the active drug and 7 of those receiving placebo[44].

As mentioned above, a known or suspected dysfunction of sphincter of Oddi is an independent risk factor for post-ERCP pancreatitis[29,46,47]. The term sphincter of Oddi dysfunction is used to define motility abnormalities caused by stenosis or dyskinesia of the sphincter of Oddi. Both alterations can account for obstruction to flow through the sphincter of Oddi thereby promoting retention of bile in the biliary tree and pancreatic juice in the pancreatic duct and clinical manifestations[48,49]. Dysfunction of sphincter of Oddi can be classified as biliary types Ⅰ, Ⅱ and Ⅲ and pancreatic types Ⅰ, Ⅱ and Ⅲ[50]. The features of biliary-type I include biliary-type abdominal pain, liver enzyme elevation and common bile duct dilation > 9 mm. Patients with biliary-type Ⅱ dysfunction have pain and only one of the remaining criteria, while biliary-type Ⅲ patients have only recurrent biliary-type abdominal pain. Patients with pancreatic-type I dysfunction have pancreatic-type abdominal pain and a dilated pancreatic duct and elevated pancreatic enzymes, or recurrent acute pancreatitis. Pancreatic-type Ⅱ dysfunction is defined as pancreatic-type abdominal pain and a dilated pancreatic duct or elevated pancreatic enzymes, while type Ⅲ patients have recurrent pancreatic-type pain alone. Elmunzer *et al*[44] showed that indomethacin was effective in preventing post-ERCP pancreatitis regardless of whether patients had sphincter of Oddi dysfunction and that the treatment was protective in all the 3 subtypes of sphincter of Oddi dysfunction. However, 82% (495/602) of patients enrolled into the trial had clinical suspicion of sphincter of Oddi dysfunction and more than half of them had manometric alterations compatible with sphincter hypertension. Since this high rate of sphincter of Oddi dysfunction is not representative of most ERCP populations, further experimentation should be performed on large numbers of patients with no sphincter of Oddi dysfunction in order to ascertain whether indomethacin is really effective in this subset of patients. The protective effect of indomethacin did not appear to be influenced by the necessity of performing pancreatic stenting, as well as by additional patient-related (age < 45 years, female gender, history of post-ERCP pancreatitis, history of recurrent pancreatitis), procedure-related (i.e., difficult cannulation, precut sphincterotomy, pancreatic sphincterotomy, pancreatic acinarization, biliary sphincterotomy, ampullectomy) and operator-related (i.e., trainee involved in stanting) characteristics. Other issues which need to be clarified relate to the time of administration and dose of the drug. The Elmunzer's study shows that the number of patients who need to be treated with indomethacin to prevent one episode of pancreatitis is 13[44]. This could reflect the fact that the time of administration was not appropriate. The indomethacin suppositories were administered immediately after ERCP[44]. Plasmatic peak of NSAID concentrations occurs within 30 min from rectal administration, a time-frame which could be sufficient to trigger pancreatitis by ERCP. Previous studies have documented a positive result in post-ERCP pancreatitis reduction even when rectal indomethacin was administered immediately pre-procedure[15,41]. We can thus speculate that a double administration of the drug, both before and after ERCP, may be more effective than a single administration. Moreover, dose-response studies would also be necessary as so far all the studies have been performed using 100 mg of drug.

CONCLUSION

Data of the present study advance our understanding of how we can prevent post-ERCP pancreatitis, even though additional studies are needed to optimize the dose and time of administration of indomethacin and verify whether the prophylactic effect of the drug can be generalized to all patients with elevated baseline risk of pancreatitis or restricted to particular subgroups.

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GUIDELINES FOR CLINICAL PRACTICE

Mirizzi syndrome: History, current knowledge and proposal of a simplified classification

Marcelo A Beltrán

Marcelo A Beltrán, Department of Surgery, La Serena Hospital, La Serena 1700000, Chile
Author contributions: Beltrán MA performed the bibliographical search and wrote the article.
Correspondence to: Marcelo A Beltrán, MD, Department of Surgery, La Serena Hospital, La Serena IV Región, La Serena 1700000, Chile. beltran_01@yahoo.com
Telephone: +56-51-485923 Fax: +56-51-333382
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Abstract

Chronic complications of symptomatic gallstone disease, such as Mirizzi syndrome, are rare in Western developed countries with an incidence of less than 1% a year. The importance and implications of this condition are related to their associated and potentially serious surgical complications such as bile duct injury, and to its modern management when encountered during laparoscopic cholecystectomy. The pathophysiological process leading to the subtypes of Mirizzi syndrome has been explained by means of a pressure ulcer caused by an impacted gallstone at the gallbladder infundibulum, leading to an inflammatory response causing first external obstruction of the bile duct, and eventually eroding into the bile duct and evolving to a cholecystocholedochal or cholecystohepatic fistula. This article reviews the life of Pablo Luis Mirizzi, describes the earlier and later descriptions of Mirizzi syndrome, discusses the pathophysiological process leading to the development of these uncommon fistulas, reviews the current diagnostic modalities and surgical approaches and finally proposes a simplified classification for Mirizzi syndrome intended to standardize the reports on this condition and to eventually develop a consensual surgical approach to this unexpected and seriously dangerous condition.

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INTRODUCTION

Chronic complications of symptomatic gallstone disease, such as Mirizzi syndrome, are rare in Western developed countries with an incidence of less than 1% a year[1]. These currently bizarre complications, are being encountered in 1% to 2% of patients with symptomatic cholelithiasis according to some recent series[1,2]. However, in underdeveloped countries; particularly in Latin-America, Mirizzi syndrome is a more common condition with a reported incidence ranging from 4.7% to 5.7%[3,4]. The importance and implications of this condition are related to their associated, and potentially serious, surgical complications such as bile duct injury, and to its modern management when encountered during laparoscopic cholecystectomy[5,6]. The pathophysiological process leading to the subtypes or stages of Mirizzi syndrome, has been explained as an inflammatory phenomenon secondary to a pressure ulcer caused by an impacted gallstone at the gallbladder infundibulum. The impacted gallstone together with the inflammatory response, causes first external obstruction of the bile duct, and eventually erodes into the bile duct evolving into a cholecystocholedochal or cholecystohepatic fistula with different degrees of communication between the gallbladder and bile duct[4,7,8].

Based on this knowledge, and after an extensive experience treating this uncommon condition, a new concept regarding Mirizzi syndrome and gallstone ileus have been developed relating both conditions and modifying the classic Csendes classification[4,7,8].

WHO WAS PABLO LUIS MIRIZZI?

Pablo Luis Mirizzi was a surgeon from Argentina, born in the city of Cordoba on January 25, 1893 and deceased in the same city on August 28, 1964 (Figure 1). Mirizzi, graduated from the Medical Sciences School at the National University of Cordoba in 1915; and in 1916 at 23 years of age defended his "Tesis Doctoral" entitled "Anemia Esplénica Cirrógena". Mirizzi was a brilliant young doctor studying under the most prominent Argentinean surgeons of their time. At the age of 25 years; in 1918, he was appointed Assistant Professor of Surgery at the same university where he had studied. During the next few years, Mirizzi traveled abroad and visited the most important and renowned surgical centers of his time in the United States of America and Europe. After he returned to Argentina, he developed his professional, scientific and academic career and in 1926 he became Full Professor of Surgery[9,10]. On June 18, 1931 Mirizzi performed the first operative cholangiography which is considered his main contribution to modern biliary surgery[9-11]. The first description made by Mirizzi compatible with the syndrome that bears his name, was published in 1940 in an article dealing with a "physiologic sphincter of the hepatic duct"[12]; The most known and cited description of his syndrome was published in 1948 postulating the functional spasm of the "common hepatic duct sphincter", which he believed existed within the common hepatic duct, as the cause of the obstructive jaundice in patients with chronic or acute gallstone disease[13]. The syndrome that Mirizzi described, consisted of an uncommon and benign cause of obstructive jaundice, caused by a gallstone impacted at the Hartmann's pouch, gallbladder infundibulum or the cystic duct and its associated inflammatory process, thereby causing obstruction of the common hepatic duct[9,10,12,13]. Mirizzi, believed that the obstruction of the common hepatic duct was rather functional, and that the mechanical obstruction of the gallbladder infundibulum and consequent inflammatory process predisposed to the contraction of a "muscular sphincter" located in the common hepatic duct[9,10,12-14]. However, current knowledge has dismissed the presence of any sphincter, or even smooth muscle, in the common hepatic duct[10,14,15]. Pablo Luis Mirizzi was recognized during his lifetime as a great surgeon, a great teacher, and a great humanitarian physician. In 1955 he was named Honorary Professor of the National University of Cordoba, and in 1956, the "Sociedad Argentina de Cirugía" honored him the title "Cirujano Maestro". Afterwards, numerous surgical academies and societies around the world awarded him honorary memberships as well as many other honors. In 1957, the Assembly of the International Society of Surgery named



Figure 1 Professor Pablo Luis Mirizzi (1893-1964).

Professor Mirizzi as President of the International Surgical Congress that was to be celebrated in Munich in 1959; this was the first time that a Latin-American surgeon had received such a high honor. Pablo Luis Mirizzi died in his city, Cordoba, on August 28, 1964. Besides his great contributions to surgery, he left his art collection to the "Museo Provincial de Bellas Artes Emilio Caraffa", donated his medical library to the Medical Sciences School of the National University of Cordoba, and established a biennial traveling surgical scholarship in biliary surgery to be awarded to distinguished young surgeons from Cordoba, Argentina[9,10].

EARLY AND LATEST DESCRIPTIONS OF THE "MIRIZZI SYNDROME"

The most common complications of chronic gallstone disease are acute cholecystitis, acute pancreatitis and acute cholangitis[1]. Other benign complications are unusual and include Mirizzi syndrome and gallstone ileus among others[1,8]. The bizarre complications of gallstone disease described in the surgical literature of the late XIX century and early XX century are seldom found today due to the widespread use and availability of ultrasonography, which have led to early diagnosis and early treatment for patients with gallstone disease, consequently avoiding the development of those late complications[4,8]. Professor Mirizzi, however, was not the first to describe the characteristics of benign obstruction of the bile duct with resultant jaundice. Partial duct obstruction secondary to an impacted gallstone and its associated inflammatory process was first described by Kehr[16] in 1905 and by Ruge[17] in 1908. Mirizzi[12] reported his syndrome for the first time in 1940, and in 1941 Levrat *et al*[18] reported other cases. Finally the famous article that established the eponym of Mirizzi[13] for this condition was published in 1948. At the same time, other authors began to report other forms of complications related to long-standing gallstone disease, and in 1942, Puestow[19] reported the first fistula between the gallbladder and the choledochus in a series of 16 patients with "spontaneous internal biliary fistulas", he described fistulas between the gallbladder and other abdominal and thoracic organs such as stomach, duodenum, colon and bronchus.

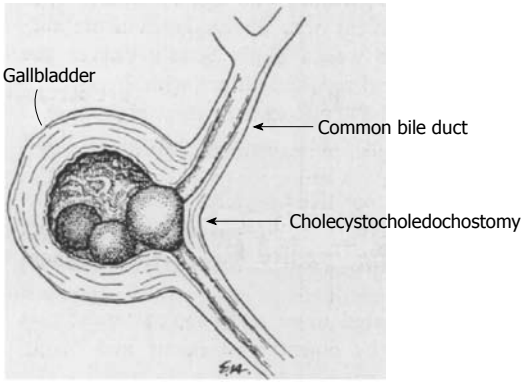


Figure 2 Albert Behrend described this case that corresponded to Mirizzi syndrome type Ⅳ according to the Csendes classification. The gallstone surrounded by an inflammatory process was clearly dilating the cystic duct fusing the gallbladder to the bile duct (original drawing from *Annals of Surgery* 1950; 132, page 300)[20].

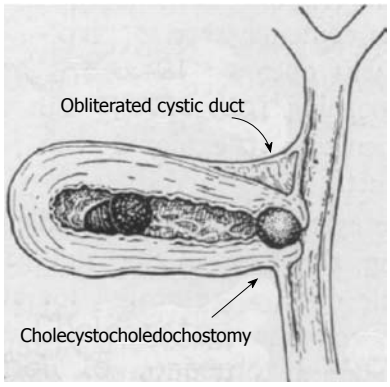


Figure 3 Another case described by Behrend. The cystic duct has become obliterated due to a chronic inflammatory process and the gallstones have eroded the gallbladder wall and formed a cholecystobiliary fistula corresponding to Mirizzi syndrome type Ⅱ according to the Csendes classification (original drawing from *Annals of Surgery* 1950; 132, page 300)[20].

Another important and overlooked article was published in 1950 by Behrend *et al*[20], in this publication they described 3 patients with cholecystobiliary fistulas; the first patient had a gallstone eroding through a much dilated cystic duct (Figure 2). The second patient had a fistula between the gallbladder wall and the choledochal wall with the cystic duct obliterated (Figure 3). The third patient had a double fistula between the gallbladder and the common hepatic duct, and between the gallbladder and the duodenum through the gallbladder wall (Figure 4), constituting the first description of what is currently known as Mirizzi Syndrome type V[4]. Soon, other authors described other cases of cholecystobiliary fistulas, Patt *et al*[21] in 1951 reported two cases, also Mirizzi[22] in 1952 reported on another four cases, and by 1975 Corlette *et al*[23] reported on another 24 cases. In 1982 McSherry *et al*[24] and in 1989 Csendes *et al*[7], published seminal articles describing the pathophysiological process involved in the development of Mirizzi syndrome, including the initial external compression of the bile duct by impacted gallstones until the erosion of

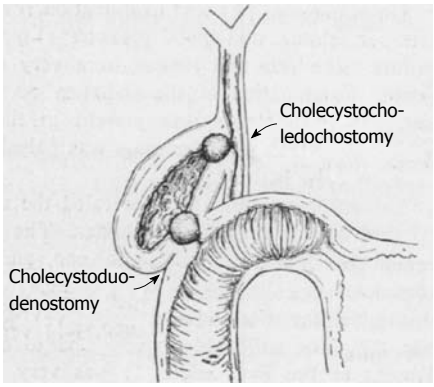


Figure 4 This illustration by Behrend clearly depicts Mirizzi syndrome type Ⅴ according to Csendes. The continuous inflammatory process affecting the gallbladder has obliterated the cystic duct, and the gallstones compressed inside the gallbladder, have produced pressure ulcers over the gallbladder walls eroding into the bile duct and the duodenum (original drawing from *Annals of Surgery* 1950; 132, page 300)[20].

the gallstones through the gallbladder wall into the bile duct. Both authors proposed similar classifications for this syndrome. Recently, the Mirizzi syndrome classification originally described by Csendes *et al*[7] was expanded to include bilioenteric fistulae; this addition was recently validated by Beltran *et al*[4].

PATHOPHYSIOLOGY

The current concept of Mirizzi syndrome, includes the external compression of the bile duct and the later development of cholecystobiliary and cholecystoenteric fistulas as different stages of the same disease process[4,7,8]. Mirizzi syndrome can be caused by an acute or chronic inflammatory condition secondary to a single large gallstone or multiple small gallstones impacted in the Hartmann's pouch or in the gallbladder infundibulum and cystic duct[1,4,7,8,14,25-37]. A long cystic duct; parallel to the bile duct, and a low insertion of the cystic duct into the bile duct, have been regarded as predisposing factors for the development of Mirizzi syndrome[1,25,31,32]. Recurrent impaction of gallstones will lead to repeated episodes of acute cholecystitis and will render the gallbladder initially distended with thick inflamed walls. Eventually the gallbladder will become contracted and atrophic, having thicker fibrotic walls if contracted. When the gallbladder becomes atrophic, it will degenerate into thick or thin atrophic walls; and in some cases the walls would be intimately adherent to the contained gallstones[1,2,25,27,37]. The close proximity of the acutely or chronically inflamed gallbladder to the bile duct could eventually lead to the fusion of their walls by edematous inflammatory tissue that in time will become fibrotic contributing to the external compression of the bile duct and leading to the characteristic obstructive jaundice seen with this condition[1,2,7,8,37], this late process could be acute or chronic[1,4,7,8,14,25-37].

The cholecystobiliary fistula has been explained by two mechanisms. The first mechanism, proposes that the

impacted gallstone and its secondary inflammatory process will lead to complete obliteration of the cystic duct, the impacted gallstone seeking to pass into the bile duct will develop a pressure ulcer that ultimately will erode the gallbladder wall and bile duct wall forming a communication between both lumens[1,8,23,27,37]. The second mechanism proposes that the impacted gallstone at the gallbladder infundibulum progressively dilates the cystic duct, leading to shortening, contraction and fibrosis of this duct and finally forming a large communication between the gallbladder and bile duct[2,23,27,37]; and even fusing the gallbladder to the adjacent bile duct[14,27,37]. If the inflammatory process continues or if a chronic inflammatory process becomes established, the gallstones will cause a pressure ulcer and necrosis, eroding into the bile duct and producing a cholecystobiliary fistula[1,2,4,8,23,27,37]. In some cases, the gallstones will also cause other pressure ulcers and concomitantly with the cholecystobiliary fistula a cholecystoenteric fistula will form[2,4,8,20].

Some characteristics of this distorted anatomy can be consistently found in Mirizzi syndrome. Consequently, based on over 70 years of publications on the subject, we may describe certain "anatomy" of Mirizzi syndrome. First, an atrophic gallbladder with either thick or thin walls[25,26,37], with impacted gallstones at the infundibulum or at the Hartmann's pouch, occasionally found firmly attached to the gallbladder wall[26,30,32,37]. Second, an obliterated cystic duct which is frequently found[2,25,30,37]. Third, a long cystic duct running parallel to the common bile duct with low insertion which has been described as a risk factor for Mirizzi syndrome[1,25,26,30,31,34]. Fourth, another anatomic variation thought to predispose to Mirizzi syndrome has been related to a normal short cystic duct[2,26,30]. Fifth, partial obstruction by external compression of the bile duct or by a gallstone eroding into the bile duct originating from the gallbladder. Sixth, a normal caliber distal bile duct with normal thickness walls[2,25,32]. Seventh, a dilated proximal bile duct with thick inflamed walls[2,25,32]. Eighth, an anomalous communication between the gallbladder and the bile duct[28]. Finally, an anomalous communication between the gallbladder and the stomach, duodenum, colon, or other abdominal viscera[4,8,28] (Table 1).

SYMPTOMATOLOGY AND OTHER CLINICAL MANIFESTATIONS

Patients with Mirizzi syndrome present with a mean age varying from 53 to 70 years, and are female with a frequency of around 70% of all cases[2,4,5,7,25,26,28,32,36]. However, it may occur at any age and in any patient with gallstones[4,27,32]. Long-standing gallstone disease, with a median of 29.6 years of disease, has been reported for patients with Mirizzi syndrome[4].

Mirizzi syndrome frequently presents in an acute form; however, the chronic form is equally or an even more common form of presentation[35]. Although the clinical presentation of Mirizzi syndrome is unspecific, the most common form of clinical presentation of Mirizzi syndrome is obstructive jaundice (60%-100%), accompanied by abdominal pain over the right upper abdominal quadrant (50%-100%), and fever in the context of a patient with known or suspected gallstone disease[1,14,26-33,35-37]. A previous recent history of jaundice can sometimes be elicited. Frequently, patients with Mirizzi syndrome present in the setting of acute cholecystitis, acute cholangitis or acute pancreatitis[1,2,27,28,33,35]. Recently, Mirizzi syndrome in the setting of gallstone ileus has been described and validated as another clinical presentation that surgeons must bear in mind[4,8].

The most common laboratory finding present in these patients is hyperbilirubinemia. Other laboratory abnormalities are elevated aminotransaminase levels and leukocytosis, frequently seen when acute cholecystitis, cholangitis or pancreatitis is present[1,25,26,28,29,33,35]. Recently, extremely high levels of the malignancy marker, CA19-9, have been consistently found in patients with Mirizzi syndrome type II or higher[38-40]. Consequently, the CA19-9 must be interpreted cautiously in patients with suspected biliary malignancy, because if Mirizzi syndrome is not ruled out, the patient would be incorrectly labeled as having biliary tract or gallbladder cancer and could miss the opportunity for curative surgery.

The differential diagnosis of Mirizzi syndrome includes any other benign or malignant cause of obstructive jaundice, such as gallbladder cancer, cholangiocarcinoma, pancreatic cancer, sclerosing cholangitis, metastatic disease, and others[1,27,31,37].

DIAGNOSIS

Preoperative diagnosis of Mirizzi syndrome followed by thoughtful surgical planning is of utmost importance[6]. The incidence of bile duct injuries in patients operated on with Mirizzi syndrome without preoperative diagnosis could be as high as 17%[14]. Preoperative diagnosis of Mirizzi syndrome is difficult and can be made in only 8% to 62.5% of patients[26,29,37,41]. If preoperative diagnosis is not made, intraoperative recognition and proper management is essential. Inadequate recognition of this condition leads to high preoperative morbidity and mortality[14]. The diagnosis of Mirizzi syndrome is based in the clinical characteristics previously described and a high index of suspicion or surgical intuition, which might be complemented by radiological images and endoscopic procedures.

Ultrasonography

A contracted gallbladder with thick or extremely thin walls with one large gallstone or multiple smaller gallstones impacted in the infundibulum may be appreciated[32]. The hepatic duct would be dilated in its extra and intrahepatic portions above the level of the obstruction site, and the common bile duct would be within normal size under the level of obstruction[1,14,28,32]. The reported diagnostic accuracy for ultrasonography in Mirizzi syn-

Table 1 Mirizzi syndrome "anatomy"

Gallbladder	Cystic duct	Bile duct	Fistulas
Thick or thin atrophic walls[25,26,37]	Obliterated cystic duct[2,25,37]	Partial obstruction by external compression or by a gallstone eroding into the bile duct	Cholecystocholedochal fistula[28]
Impacted gallstones at the infundibulum or at the Hartmann's pouch[26,30,32,37]	Occasional absence of cystic duct[2,25,30]	Normal caliber distal bile duct with walls of normal thickness[2,25,32]	Cholecystoenteric fistula[4,8,28]
	A long cystic duct running parallel to the common bile duct with low insertion[1,25,26,30,31,34]	A dilated proximal bile duct with thick inflamed walls[2,25,32]	
	A normal short cystic duct[2,26,30]		

drome is 29%[29], with a reported sensitivity varying from 8.3% to 27%[33,41].

Computed tomography

Abdominal computed tomography (CT) can identify the gallbladder and measure its wall thickness and the bile duct dilatation. However, the presence of periductal inflammation can be misinterpreted as gallbladder cancer[27]. The radiological signs elicited by CT are not specific. The main utility of CT would be the exclusion of malignancy in the porta hepatis area or in the liver[1,14,32].

Magnetic resonance

Cholangioresonance or magnetic resonance cholangiopancreatography (MRCP) is a useful tool to demonstrate extrinsic compression of the bile duct and to determine whether a fistula is present or not. Moreover, it is useful to rule out choledocholithiasis and other causes of bile tract obstruction. Some typical features of Mirizzi syndrome can be shown by MRCP such as the extrinsic narrowing of the common hepatic duct, a gallstone in the cystic duct, dilatation of the intrahepatic and common hepatic ducts, and a normal choledochus. Magnetic resonance imaging can also show the extent of the inflammatory process surrounding the gallbladder and has the advantage of avoiding the complications associated with endoscopic cholangiography[14]. However, the diagnostic accuracy for MRCP is 50%[29].

Endoscopic retrograde cholangiopancreatography

Endoscopic retrograde cholangiopancreatography (ERCP) is an invasive procedure not only useful to confirm the presence of Mirizzi syndrome with or without cholecystobiliary or cholecystoenteric fistulae, but also for therapeutic means allowing stone retrieval, stent placement and other procedures[1,14,28,41]. The diagnostic accuracy of Mirizzi syndrome with this method reaches around 55% to 90%[29,41], with a failure rate ranging from 5% to 10%[33,41]. The features of ERCP in Mirizzi syndrome include a narrowing or curvilinear extrinsic compression involving the lateral portion of the distal common hepatic duct with proximal ductal dilatation and normal distal caliber[14,27,32,41].

Intraoperative diagnosis

Over 50% of patients with Mirizzi syndrome are diagnosed during surgery[29]. Surgical characteristics include the presence of a shrunken gallbladder with distorted anatomy or a dilated gallbladder with thick walls and a large stone, or multiple gallstones, impacted at the gallbladder neck or infundibulum, an obliterated Calot's triangle, a dense fibrotic mass at the Calot's triangle, and dense adhesions at the subhepatic space. Intraoperative cholangiography could be useful and help to confirm the diagnosis, determine the location and size of the fistula, detect bile duct stones, and detect whether there is any loss of integrity of the bile duct wall[14]. However, intraoperative cholangiography may be difficult to perform and persistent dissection at the Calot's triangle area may result in a bile duct injury. Intraoperative ultrasonography has been reported to be a useful tool to identify the anatomy of the biliary tree and help with an accurate dissection of the bile duct in an inflamed area[14,28].

GALLSTONE ILEUS AND MIRIZZI SYNDROME

The first description of gallstone ileus was made by the notable anatomist Thomas Bartolin in 1654 as a disease of elderly and debilitated people. In 1890 Courvoisier[42] described 131 cases with a surgical mortality rate approaching 50%. Gallstone ileus is classically described as the impaction of one or more gallstones in the small bowel, frequently at the distal ileum[43]. However, an interesting form of gallstone ileus is the obstruction of the duodenum by an impacted gallstone causing gastric retention syndrome, first described in 1770 by Beaussier, and later by Bonnet in 1841 and by Bouveret[44] in 1896. Gallstone ileus accounts for 1% to 4% of all mechanical small bowel obstructions and for up to 25% of small bowel obstructions in patients older than 65 years, with a female preponderance[43]. The pathophysiology of gallstone ileus resembles that of Mirizzi syndrome. An episode of acute cholecystitis triggers a chronic inflammatory process causing a pressure ulcer by the offending gallstones and eventually eroding through the gallbladder wall into the bowel wall forming a cholecystoenteric fistula and allowing the passage of a single or multiple gallstones[1,2,4,7,8]. These similar pathological processes between cholecystoenteric fistula and Mirizzi syndrome with cholecystocholedochal fistula, were related in 2005 by Beltrán *et al*[8], constituting the basis for the addition by Csendes of a

Table 2 Mirizzi syndrome milestones and historical classification

Author	External compression of common hepatic duct	Cholecystocholedochal fistula	Cholecystoenteric fistula
Puestow[19], 1942	-	Mirizzi Ⅱ	-
Mirizzi[13], 1948	Mirizzi Ⅰ	-	-
Corlette *et al*[23], 1975	-	Mirizzi Ⅱ (Fistula type Ⅰ and Ⅱ)	
McSherry *et al*[24], 1982	Mirizzi Ⅰ	Mirizzi Ⅱ	-
Csendes *et al*[7], 1989	Mirizzi Ⅰ	Mirizzi Ⅱ, Ⅲ, Ⅳ	-
Csendes *et al*[4], 2008	Mirizzi Ⅰ	Mirizzi Ⅱ, Ⅲ, Ⅳ	Mirizzi Va, Vb

Table 3 Incidence of the different types of Mirizzi syndrome according to Csendes 2007 classification

Type of Mirizzi	Incidence (%)[4,7,14,28,29]
Ⅰ	10.5-78
Ⅱ	15-41
Ⅲ	3-44
Ⅳ	1-4
Ⅴ	29

fifth grade to his widely known classification[7], which was later validated in 2008 by Beltrán *et al*[4]. An interesting fact regarding this relationship between Mirizzi syndrome and cholecystoenteric fistula, is that many authors have previously described Mirizzi syndrome associated to fistulas communicating the gallbladder with the duodenum, colon, stomach or jejunum, failing to describe an association between these two conditions[28,45,46].

CLASSIFICATION

Before recognition of external compression of the bile duct and cholecystobiliary fistula as different evolving stages of the same disease process during the decade of 1980; in 1975, Corlette *et al*[23] classified the cholecystocholedochal fistulas in two types. Type Ⅰ was defined when the fistula involved the Hartmann´s pouch and the bile duct. Type Ⅱ was defined when the gallstone dilated and eroded the cystic duct into the bile duct. McSherry *et al*[24] in 1982, classified the Mirizzi syndrome into two types based on ERCP findings. Type Ⅰ involves the external compression of the bile duct by a large stone or stones impacted in the cystic duct or in the Hartmann' s pouch. Type Ⅱ consists of a proper cholecystobiliary fistula, caused by a gallstone or gallstones that have eroded into the bile duct. In 1989 Csendes *et al*[7], modified the classification of McSherry by dividing Mirizzi syndrome into four types. Csendes classification (Figure 5) further categorized the cholecystobiliary fistula according to the extent of bile duct destruction. Csendes type Ⅰ corresponds to McSherry type Ⅰ, the external compression of the bile duct by an impacted gallstone in the gallbladder infundibulum or cystic duct. Mirizzi syndrome type Ⅱ consists of a cholecystobiliary fistula resulting from erosion of the bile duct wall by a gallstone, the fistula must involve less than one-third of the circumference of the bile duct. Mirizzi syndrome type Ⅲ consists of a cholecystobiliary fistula involving up to two-thirds of the bile duct circumference. Mirizzi syndrome type Ⅳ is a cholecystobiliary fistula with complete destruction of the bile duct wall with the gallbladder completely fused to the bile duct forming a single structure with no recognizable dissection planes between both biliary tree structures. In 2007, Csendes added one more type to his classification that was later validated by Beltran and Csendes in 2008[4]; the Mirizzi type Ⅴ, which includes the presence of a cholecystoenteric fistula together with any other type of Mirizzi. Furthermore, Mirizzi type Va includes a cholecystoenteric fistula without gallstone ileus and Mirizzi type Vb refers to a cholecystoenteric fistula complicated by gallstone ileus[4]. A simple historical comparison between the most well-known Mirizzi syndrome classifications is depicted in Table 2. Other lesser known classification systems have been described over the last 25 years; such as acute *vs* chronic variant, anatomic variant of cystic duct *vs* no anatomic variant of cystic duct, and obstruction due to gallstones *vs* obstruction due to inflammation[14,30,35]. The incidence of the different types of Mirizzi syndrome according to Beltrán and Csendes[4] is detailed in Table 3. Mirizzi type Ⅰ is fairly common (10.5% to 51%) and Mirizzi Ⅳ is rather uncommon (1% to 4%). Mirizzi Ⅴ, however, could be present in up to 29% patients with any other type of Mirizzi. Recently, some authors have used the new addition to the former Csendes classification to describe their patients with complex cholecystobiliary fistulas and associated cholecystoenteric fistulas[47].

TREATMENT

Patients with Mirizzi syndrome constitute a formidable challenge and their surgical treatment a test to any surgeon's ability and dexterity[8]. The treatment of Mirizzi syndrome is surgical. Mirizzi syndrome is important to surgeons because preoperative diagnosis is not always possible and because the surgical treatment of this condition is associated with a significantly increased risk of bile duct injury[48-56]. Besides, the severe inflammatory process with thick dense hard adhesions and associated edematous tissues distort the anatomy[37,48,51,53,55]. Additionally, the presence of a cholecystobiliary fistula further increases the risk of biliary duct injury[48,52,53]. During the operation, dissection of the Calot's triangle may lead to bile duct injury or excessive bleeding and other morbidity

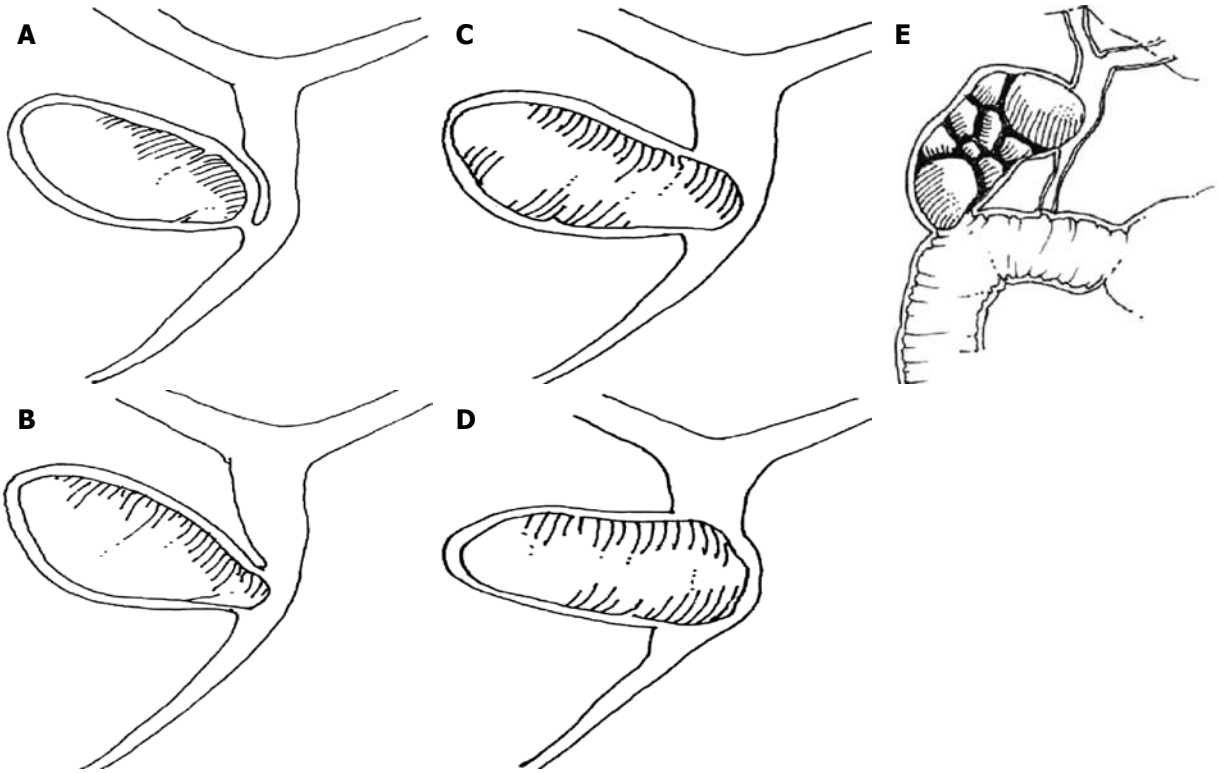


Figure 5 Mirizzi syndrome type Ⅰ to type Ⅴ. A: Mirizzi type Ⅰ is the extrinsic compression of the common bile duct by an impacted gallstone; B: Mirizzi type Ⅱ consists of a cholecystobiliary fistula involving one third of the circumference of the bile duct; C: In Mirizzi type Ⅲ, the cholecystobiliary fistula compromises up to two-thirds of the circumference of the bile duct; D: In Mirizzi type Ⅳ, the cholecystobiliary fistula has destroyed the bile duct wall, and comprises the whole circumference of the bile duct; E: Mirizzi type V corresponds to any type of Mirizzi associated with a bilioenteric fistula with or without gallstone ileus (original drawings from *World Journal of Surgery* 2008; 32, pages 2239 to 2241)[4].

such as sepsis, delayed bile duct stricture, and secondary biliary cirrhosis[14,26,28,48,53]. The surgical treatment of Mirizzi syndrome avoids a truly standardized approach and must be individualized depending on the stage of the disease and the expertise of the surgical team[55]. However, some guidelines could be drawn and have been used during the last few years.

Open surgery

Subtotal cholecystectomy may be the best treatment for Mirizzi type I and most cases of Mirizzi type Ⅱ and Ⅲ[48-57]. Subtotal cholecystectomy was described in 1985 by Bornman *et al*[58] for difficult open cholecystectomy in patients with severe cholecystitis associated with liver cirrhosis and portal hypertension. Since then, this technique has been also applied to cases of Mirizzi syndrome. Recently, subtotal cholecystectomy has been described in laparoscopic cholecystectomy, following the same technical details described for the open technique[59]. After identification of the anatomical repairs and having determined the presence of Mirizzi syndrome, the gallbladder is approached by an incision running from the fundus to the Hartmann's pouch, or if possible in selected cases, directly over the Hartmann's pouch to remove the impacted gallstone[14,26,27,32,33,37,48,49,51,54-59]. The reflux of bile indicates the presence of a fistula between the gallbladder and the bile duct, because the cystic duct is usually occluded[48-55]. If no fistula is macroscopically evident or diagnosed by intraoperative cholangiography, a partial cholecystectomy leaving the gallbladder neck or infundibulum is performed and the gallbladder stump is closed with absorbable monofilament sutures over the bile duct. However, it must be kept in mind that sometimes a classic cholecystectomy could be performed. If a fistula is present (Mirizzi Ⅲ and Ⅳ), besides partial cholecystectomy, a biliary-enteric anastomosis could sometimes be performed between the duodenum and bile duct or between the bile duct and a loop of jejunum *en-Y-de-Roux*[7,14,29,33,37,48,51,55]. The rationale for a biliary-enteric anastomosis is that because of the continued inflammation of the gallbladder flap, strictures can develop, with an even greater risk for large cholecystocholedochal fistulas or badly damaged bile ducts[55]. Baer *et al*[48] have advocated an anastomosis between the partially resected gallbladder and the duodenum which they called "cholecysto-choledocho-duodenostomy" for patients with Mirizzi Ⅱ or higher, claiming satisfactory results and decreased risk of bile duct injury. A variation of the Baer anastomosis is the "cholecysto-choledocho-jejunostomy" proposed by Safioleas *et al*[54], for patients with Mirizzi Ⅱ or higher. Others have proposed a standardized surgical approach based on the grade of Mirizzi encountered, which seems to be a logical and appropriate management; however, not always plausible[4,7,52]. The bile duct should be explored for the presence of stones through a different incision over the common hepatic duct or the choledochus, because common bile duct stones are a common

occurrence in Mirizzi syndrome and have been found in 25% to 40% of cases[26,53]. The common bile duct suture must be protected with a Kehr tube placed through a different hepaticotomy or choledochotomy proximal or preferable distal to the fistula[7,28,30,33,37,41,52]. Operative cholangiography through the Kehr tube should be performed in all patients with Mirizzi syndrome before concluding the surgical procedure[26-33,51,53].

Treatment of Mirizzi syndrome type I

Acute and chronic presentation of Mirizzi syndrome type I could be adequately resolved by classic cholecystectomy[1-37,48,50,52,53,56]. Those cases with extensive chronic or acute inflammation of Calot's triangle area could be securely operated on with subtotal cholecystectomy with excellent outcomes[48-57,59]. The approach to the gallbladder in subtotal cholecystectomy begins by choosing a convenient site, more commonly the fundus and in selected cases the Hartmann´s pouch or even over the impacted gallstone, to open the gallbladder wall[49,50,54,55,57-59]. The gallstone or gallstones are then emptied and the gallbladder is removed starting the dissection at the fundus[57]. Sometimes, the gallstones are firmly adhered to the gallbladder wall and must be extracted with mucosa or whole wall, on other occasions the gallstones are loosely attached to the wall and are easily removed. The cystic duct is identified from inside the open gallbladder and explored seeking residual gallstones. In some cases, the cystic duct is obliterated and no attempt should be made to open it. Finally, the cystic duct is secured without further dissection. If the bile duct is to be explored this must be done through a separate incision and protected by a Kehr tube[7,14,26-30,53].

Treatment of Mirizzi syndrome type II

The initial approach to patients with cholecystobiliary fistula involves a subtotal cholecystectomy starting the dissection from the gallbladder fundus towards the Hartmann´s pouch. Most cholecystobiliary fistulas are diagnosed during surgery, and not during the preoperative study. The gallbladder should be removed leaving a remnant of gallbladder wall measuring about 5 mm around the cholecystobiliary fistula in order to aid in the closure of the destroyed bile duct[52,54]. Mirizzi syndrome type II can be successfully treated with this technique[55,56]. The exploration of the bile duct should always be undertaken using a separate incision distal to the fistula and protected by a Kehr tube[30]. Placing the Kehr tube through the fistula increases the risk of bile leakage and bile duct stricture[7,14,30,50].

Treatment of Mirizzi syndrome type III

Most cases of Mirizzi syndrome type III can be treated by subtotal cholecystectomy leaving a flap of gallbladder wall measuring at least 1 cm to repair the bile duct[52,53]. However, some cases with an important inflammation of the gallbladder wall will need another procedure such as a bilioenteric anastomosis to the duodenum or a hepaticojejunostomy *en-Y-de-Roux*[7,27,36,37,48,50,51,54-56].

Treatment of Mirizzi syndrome type IV

The treatment of this type of Mirizzi with extensive destruction of the bile duct wall consists on a bilioenteric anastomosis. A hepaticojejunostomy *en-Y-de-Roux* is preferred[7,27,3,37,46,50-56].

Treatment of Mirizzi syndrome type V

Mirizzi type V can be associated with an acute serious condition or with chronic active or inactive bilioenteric fistulae; consequently the treatment differs accordingly. Mirizzi syndrome type Va should be treated with division and simple suture with an absorbable material of the bilioenteric fistulae over the implicated viscera (duodenum, stomach, colon or small bowel) and cholecystectomy, either total or subtotal according to the presence of a cholecystobiliary fistula or simple external compression of the bile duct. Mirizzi syndrome type Vb is a matter of controversy, nonetheless, it seems advisable to treat the acute condition first (gallstone ileus) and at a second time after the patient has recovered from surgery (3 or more months later), approach the gallbladder according to the presence or absence of external compression of the bile duct or cholecystobiliary fistula[8].

Laparoscopic surgery

Laparoscopic cholecystectomy can be dangerous for the bile duct[6,60,61]. Some conditions increase the risk of bile duct injuries in laparoscopic cholecystectomy: uncommon pathology such as Mirizzi syndrome, difficult or distorted anatomy, and technical problems[5,6,60,62,63]. Severe inflammation in the triangle of Calot area makes dissection of the cystic duct and cystic artery hazardous[5,6,60,62]. It has been suggested that preoperative diagnosis of Mirizzi syndrome decreases the rate of conversion and complications, thus a stronger emphasis should be given to the preoperative diagnosis of this syndrome[6]. Currently, and despite various studies reporting the feasibility of laparoscopic surgery in Mirizzi syndrome, laparoscopic cholecystectomy for this condition is considered controversial and technically challenging, placing the patient at a probably unnecessary increased risk of bile duct injuries; as a consequence, laparoscopic cholecystectomy for Mirizzi syndrome cannot currently be recommended as a standard procedure[6]. Also, besides being technically demanding, the surgical laparoscopic treatment of Mirizzi syndrome requires some technology still not widely available such as intraoperative ultrasonography, laparoscopic choledocoscopy or the use of laparoscopic staplers[4,5,62]. Furthermore, careful analysis of the reports on laparoscopic surgery for Mirizzi syndrome have revealed increased complication rates compared to open cholecystectomy[33,35]. Laparoscopic subtotal cholecystectomy and dissection of the gallbladder from the fundus towards the infundibulum have been described to reduce the risk of bile duct injury and conversion to open surgery rates[5,59,64]. Probably laparoscopic cholecystectomy could be carefully undertaken in selected patients with Mirizzi syndrome type I [5,50,61-64]. However, it is not recommended for patients with Mirizzi syndrome type II or high-

er[5,60]. Moreover, successful laparoscopic cholecystectomy in patients with Mirizzi type I can be predicted based in the visualization of the cystic duct during the initial dissection over the Calot's triangle[63]. A series reporting laparoscopic cholecystectomy in Mirizzi syndrome had a conversion rate as high as 31% to 100%, with a complication rate of 0% to 60%, bile duct injury of 0% to 22% and mortality ranging from 0% to 25%[6,28,55,56,61].

A step-by-step approach to the laparoscopic management of Mirizzi syndrome summarizing much of the current knowledge has been proposed by Rohatgi *et al*[62], highlighting the initial section of the gallbladder fundus and retrieval of the impacted gallstones to identify the gallbladder infundibulum and cystic duct from within the gallbladder and facilitate the subtotal cholecystectomy; they also emphasize the importance of operative cholangiography. The identification of some steps and associated anatomic landmarks would make laparoscopic cholecystectomy on these patients safer, these steps and anatomic landmarks have been proposed by Singh *et al*[65]. This proposal consists of the initial approach to the gallbladder that must be kept close to the liver margin, early definition of the gallbladder neck, early definition of the gallbladder-cystic duct junction, identification of the cystic lymph node as a landmark between the cystic duct and cystic artery, appropriate and careful dissection of the Calot's triangle, identification of the Rouviere's sulcus as a landmark to the plane of the common bile duct, and continuous localization and identification of the common bile duct during surgery.

ERCP

ERCP besides being diagnostic allows the performance of a sphincterotomy for stone extraction and facilitates other interventions such as the placement of stents or a nasobiliary tube or other procedures[29,35,41,49,50,51,55,56,61]. Poor surgical candidates may have an attractive option with the use of ERCP to relieve the obstruction of the bile duct caused by Mirizzi syndrome. Patients with associated cholangitis will benefit from preoperative biliary drainage as a temporary measure before a definitive operation. In general, endoscopic management includes both, biliary drainage and stone removal, and eventually stent insertion. Standard stone removal techniques are usually used and include baskets, balloons, and mechanical and electro-hydraulic lithotripsy. However, ERCP has known associated morbidity and mortality and its risks must be weighed against its benefits in patients suspected of having Mirizzi syndrome.

GALLBLADDER CANCER AND MIRIZZI SYNDROME

Mirizzi syndrome constitutes one differential diagnosis of gallbladder carcinoma. It has been reported that 6% to 28% of patients with preoperative diagnosis of Mirizzi syndrome actually had gallbladder cancer[14,61]. Gallbladder cancer has been associated with various degrees of Mirizzi syndrome with an incidence varying between 5.3% and 28% of patients[66-69]. Gallbladder cancer is found associated with Mirizzi syndrome mainly in patients with Mirizzi syndrome type Ⅱ or higher[66,68]. Moreover, patients with Mirizzi syndrome and biliary-enteric fistulas (Mirizzi type Ⅴa) associated with gallbladder cancer have been reported[66]. Mirizzi syndrome and gallbladder cancer share the same risk factor in their etiology: long-standing gallstone disease with stasis. Consequently, Mirizzi syndrome must be considered a risk factor for gallbladder cancer[67,68]. There are no clinical features to distinguish Mirizzi syndrome from gallbladder cancer, except that patients with gallbladder cancer are a decade older than patients with Mirizzi syndrome alone[67]. Measurement of the tumor marker, CA19-9, could help to differentiate between Mirizzi syndrome and gallbladder cancer, although CA19-9 is moderately elevated in benign diseases associated with obstructive jaundice including Mirizzi syndrome[38-40], this marker is markedly elevated with levels over 800 UI/mL in patients with gallbladder cancer[68]. Nonetheless, it must be kept in mind that high levels of CA19-9 have been found in some patients with Mirizzi syndrome[38-40]. Patients with suspected Mirizzi syndrome must undergo extensive preoperative study with CT and magnetic resonance cholangiography to rule out gallbladder cancer[66-69]. Tomographic findings suggesting gallbladder cancer and not Mirizzi syndrome are the presence of a mass filling or replacing the gallbladder which is seen in 40% to 60% of patients, local liver involvement, and focal protrusion of the anterior surface of the liver[66]. During the operation, frozen section biopsy must be performed in all patients with Mirizzi syndrome to definitively rule-out gallbladder cancer. If a surgically resectable gallbladder cancer is diagnosed, a radical cholecystectomy with hepatic wedge resection and lymph node dissection must be performed. The diagnosis of gallbladder cancer must be ruled out before or during surgery and an appropriate surgery must be performed because a second surgery to perform oncologic surgery has a poorer prognosis and outcome than a one-stage radical surgery[14]. Nonetheless, long-term survival in patients with Mirizzi syndrome and gallbladder cancer can be attained[67].

A PROPOSITION FOR A SIMPLIFIED CLASSIFICATION

Despite numerous attempts to standardize the surgical treatment of Mirizzi syndrome, no consensus has been reached and none of the proposed algorithms has been accepted by the world surgical community. The main reasons for the failure to establish a standard surgical approach to Mirizzi syndrome are the rarity of this condition and its unexpected presentation during surgery; these reasons preclude the design and performance of controlled randomized trials to test the proposed surgical approaches. Besides, there are no international publications in the English language reporting long-term outcomes of the surgical treatment of any type of Mirizzi syndrome.

Table 4 New proposed classification and suggested surgical treatment for Mirizzi syndrome

Type of Mirizzi	Description	Treatment
Ⅰ	External compression of the bile duct	Open cholecystectomy Open subtotal cholecystectomy Laparoscopic cholecystectomy Laparoscopic subtotal cholecystectomy
Ⅱa	Cholecystobiliary fistula < 50% the diameter of the bile duct	Open cholecystectomy Open subtotal cholecystectomy
Ⅱb	Cholecystobiliary fistula > 50% the diameter of the bile duct	Open subtotal cholecystectomy Biliary-enteric derivation: Side-to-side to the duodenum *En-Y-de-Roux* to the Jejunum
Ⅲa	Cholecystobiliary fistula and cholecystoenteric fistula without gallstone ileus	Simple closure of the fistula and treatment of the gallbladder according to the presence of Mirizzi Ⅰ, Ⅱa or Ⅱb
Ⅲb	Cholecystobiliary fistula and cholecystoenteric fistula with gallstone ileus	Treatment of the gallstone ileus and deferred treatment of the gallbladder according to the presence of Mirizzi Ⅰ, Ⅱa or Ⅱb

Consequently, no conclusions can be drawn regarding the real utility of the currently used and proposed surgical techniques for Mirizzi syndrome.

The importance and ultimate goal of all classification systems for Mirizzi syndrome is to enable the surgeon to tailor the surgical approach to the individual case[61]. This goal has not been entirely fulfilled by the current published classifications. Moreover, the recognition of cholecystoenteric fistulae associated with Mirizzi syndrome and the consequent addition of a new grade of Mirizzi to the classic classification of Csendes has further complicated the correct diagnosis, classification, and treatment of patients with Mirizzi syndrome. In 2009, Solis-Caxaj suggested that the classification of Mirizzi syndrome should be simplified taking into account the recent addition by Csendes[70], he proposed that the classification should have only 3 types. The proposition of Solis-Caxaj makes sense and given the current knowledge on Mirizzi syndrome, which is well established, I believe that a more simple classification should be proposed as follows: Mirizzi Ⅰ, external compression of the bile duct by a chronic or acute inflammatory process and a gallstone impacted at the Hartmann pouch or at the gallbladder infundibulum. Mirizzi Ⅱa, cholecystobiliary fistula less than 50% the diameter of the bile duct. Mirizzi Ⅱb, cholecystobiliary fistula more than 50% the diameter of the bile duct. Mirizzi Ⅲa, cholecystobiliary fistula associated with a concurrent cholecystoenteric fistula without gallstone ileus. Mirizzi Ⅲb cholecystobiliary fistula associated with a concurrent cholecystoenteric fistula with gallstone ileus. Based on this proposed classification and the published world-wide experience, a standardization of the surgical treatment could be suggested (Table 4).

CONCLUSION

Mirizzi syndrome continues to be a fascinating subject of study, this fascination arises due to the challenge it represents and the unexpectedness of its presentation complicating an assumedly simple surgery as some currently consider being the cholecystectomy. Even though more than 60 years have passed since the description of Pablo Luis Mirizzi, it seems that the last word on this subject is yet to be pronounced. The approach to patients with suspected Mirizzi syndrome should be cautious and sound. Every effort should be made to establish a preoperative correct diagnosis, and if encountered during surgery every effort should be made to perform an accurate and cautious surgery trying to identify the type of Mirizzi and perform the most adequate treatment for each particular case. This article extensively reviews the current knowledge on Mirizzi syndrome and based on this knowledge proposes a simplified classification suggesting the surgical approach according to each type of Mirizzi.

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GUIDELINES FOR CLINICAL PRACTICE

Interactions between hepatic iron and lipid metabolism with possible relevance to steatohepatitis

Umbreen Ahmed, Patricia S Latham, Phillip S Oates

Umbreen Ahmed, Department of Physiology, Army Medical College, National University of Sciences and Technology, Rawalpindi 46000, Pakistan
Patricia S Latham, Department of Pathology, The George Washington School of Medicine and Health Sciences, Washington, DC 20037, United States
Phillip S Oates, Physiology M311, Anatomy, Physiology and Human Biology, University of Western Australia, Perth, WA 6009, Australia
Author contributions: All authors contributed equally to the production of this manuscript.
Correspondence to: Dr. Phillip S Oates, Physiology M311, Anatomy, Physiology and Human Biology, University of Western Australia, Perth, WA 6009, Australia. poates@cyllene.uwa.edu.au
Telephone: +61-8-64881391 Fax: +61-8-64881025
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Abstract

The liver is an important site for iron and lipid metabolism and the main site for the interactions between these two metabolic pathways. Although conflicting results have been obtained, most studies support the hypothesis that iron plays a role in hepatic lipogenesis. Iron is an integral part of some enzymes and transporters involved in lipid metabolism and, as such, may exert a direct effect on hepatic lipid load, intrahepatic metabolic pathways and hepatic lipid secretion. On the other hand, iron in its ferrous form may indirectly affect lipid metabolism through its ability to induce oxidative stress and inflammation, a hypothesis which is currently the focus of much research in the field of non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH). The present review will first discuss how iron might directly interact with the metabolism of hepatic lipids and then consider a new perspective on the way in which iron may have a role in the two hit hypothesis for the progression of NAFLD *via* ferroportin and the iron regulatory molecule hepcidin. The review concludes that iron has important interactions with lipid metabolism in the liver that can impact on the development of NAFLD/NASH. More defined studies are required to improve our understanding of these effects.

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Key words: Lipids; Iron; Non-alcoholic fatty liver disease/non-alcoholic steatohepatitis; Hepcidin; Oxidative stress

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ROLE OF IRON IN HEPATIC LIPID METABOLISM: A BRIEF OVERVIEW

The liver is a major site for the storage of iron and the metabolism of lipids and is therefore an important site for interaction between these two metabolic pathways. One role of iron in the pathogenesis of diseases associated with hyperlipidemia and lipid deposition is likely to include an ability to induce oxidative stress and inflammation in the liver. In the setting of lipid deposition in the liver, iron overload has been associated with increased fibrosis and progression of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (NAFLD/NASH)[1-3]. Iron in its ferrous form can generate free radicals *via* the Fenton reaction, resulting in oxidative stress and lipid peroxidation. These effects can, in turn, stimulate hepatic

stellate cells to increase production of collagen and progression of fibrosis in patients with NAFLD and NASH. Notwithstanding these indirect effects, there are also recent and archival studies to show that iron has direct effects on lipid metabolism. Variations in hepatic iron stores result in inappropriate lipogenesis for lipid storage and secretion. For example, iron deficiency is associated with increased hepatic lipogenesis and lipemia[4,5]. Since the liver co-ordinates iron and lipid metabolism, and under conditions of stress is the site of excessive deposition of both these nutrients, this review will focus on the role of iron in the metabolism of hepatic lipids. In particular, it examines the role of different enzymes, receptors and transporters, as well as the effects of proteins involved in lipogenesis and lipoprotein secretion as sites for interaction between iron and lipids. The review then addresses the way in which iron may function in the progression of NAFLD. To begin, a brief overview of normal hepatic lipid metabolism is provided.

HEPATIC LIPID METABOLISM

Hepatic steatosis occurs whenever there is an imbalance among the uptake, synthesis, oxidative and secretory pathways of fatty acid metabolism. Sources of fatty acids for hepatic triglyceride (TG) synthesis are those derived from the plasma non-esterified fatty acids (NEFA) pool and those from within the liver through *de novo* lipogenesis (DNL), mainly from glucose (Figure 1). The hepatic uptake of fatty acid from the plasma is not regulated and is therefore directly related to the concentration of plasma NEFAs. Adipose tissue by its TG lipolysis is the main contributor to the plasma NEFA pool. The remaining NEFAs come from peripheral lipolysis of lipoproteins [chylomicrons (CM), very low density lipoprotein (VLDL)] and dietary fatty acids in the circulation. The hepatic uptake of fatty acids occurs by fatty acid binding protein and fatty acid translocase. Once in the hepatocyte, fatty acids undergo oxidation or they may be detoxified by re-esterification with glycerol and cholesterol to form TG and cholesteryl esters (CE), respectively. The TG and CE are then secreted as VLDL into the plasma or, if in excess, are stored in the cytoplasm as lipid droplets. Adipose differentiation-related protein (ADRP) is expressed in hepatocytes and is involved in cytosolic lipid storage[6].

The partitioning of hepatic TG into different pools under normal conditions of fasting/feeding and dietary variations, i.e. carbohydrate or fat diet, involves different hormonal and metabolic regulatory mechanisms. Insulin is a fat-sparing hormone and a major metabolic factor in the regulation of fat metabolism. In the liver, insulin increases TG synthesis by the hepatocytes and restricts its secretion by partitioning the newly synthesized TG into cytosolic stores[7]. In addition, insulin also inhibits lipolysis in the hepatocyte[8], decreases the assembly of VLDL by impairing the association of apoB-100 with TG[9], inhibits the translation of apoB-100 mRNA in human hepatocellular carcinoma (HepG2) cells[10] and stimulates the degradation of newly synthesized apoB[11], which collectively decrease VLDL-TG secretion. These actions coupled with insulin-stimulated expression of ADRP promotes lipid droplet assembly[12]. Furthermore, insulin also activates sterol regulatory element binding protein-1c, which transcriptionally activates nearly all genes involved in DNL[13].

EFFECTS OF IRON DEFICIENCY ON HEPATIC LIPIDS

There is evidence to suggest that lipoprotein uptake pathways are affected by iron.

Anaemia/hypoxia is associated with decreased fat uptake by the liver, but increased fat accumulation by the liver. Decreased fat uptake may either be due to impaired peripheral lipolysis or decreased uptake of CM and VLDL remnants, leaving the lipoproteins to accumulate in the circulation. Rat models of chronic anemia/hypoxia in which iron deficiency is present show decreased lipoprotein lipase and hepatic lipase activities[14-18]. This may be due to decreased synthesis[18], defects in the structure or conversion from precursor to the functional form, or in the activation of these enzymes[14,16]. It may be that iron itself can impact on hepatic uptake pathways. Supporting this idea, Brown *et al*[19] have shown that the binding of low density lipoprotein to its receptors requires a divalent cation. Although speculative, one of the divalent cations may include ferrous-iron. Anaemic rats produced by experimentally induced chronic renal failure show down-regulation of the expression of the VLDL-receptor in a non-erythropoietin dependent manner[20]. Furthermore, the plasma ratio of cholesteryl ester to cholesterol is increased with iron deficiency in rats. This cannot be explained by increased lecithin cholesterol acyl transferase (LCAT) activity, since LCAT is reduced in iron deficiency[21,22].

On the other hand, iron deficiency in rats has been shown to increase hepatic lipogenesis, leading to cellular TG accumulation and steatosis[4,5,23,24]. Given that hepatic uptake pathways are down regulated, the increased lipogenesis could be due to decreased utilization of fatty acids or increased DNL. In iron deficient rats, it is proposed that beta-oxidation of fatty acids is decreased, allowing the fatty acids to be diverted towards TG synthesis[24]. Supporting this, the level of hepatic carnitine, the long chain fatty acid transporter required for β-oxidation of fatty acids is reduced in the iron deficient state. Since this enzyme requires iron as a cofactor, its activity might be expected to decrease with iron deficiency[24,25]. However, others report that beta-oxidation of fatty acids remains unchanged[4], while hepatic DNL increases[4,23]. The increased hepatic lipogenesis seen in iron-deficient rats is supported by genomic studies that show a concurrent decrease in gene expression related to hepatic β-oxidation and an increase in gene expression related to lipogenesis[26]. Therefore, some of the changes in lipid metabo-

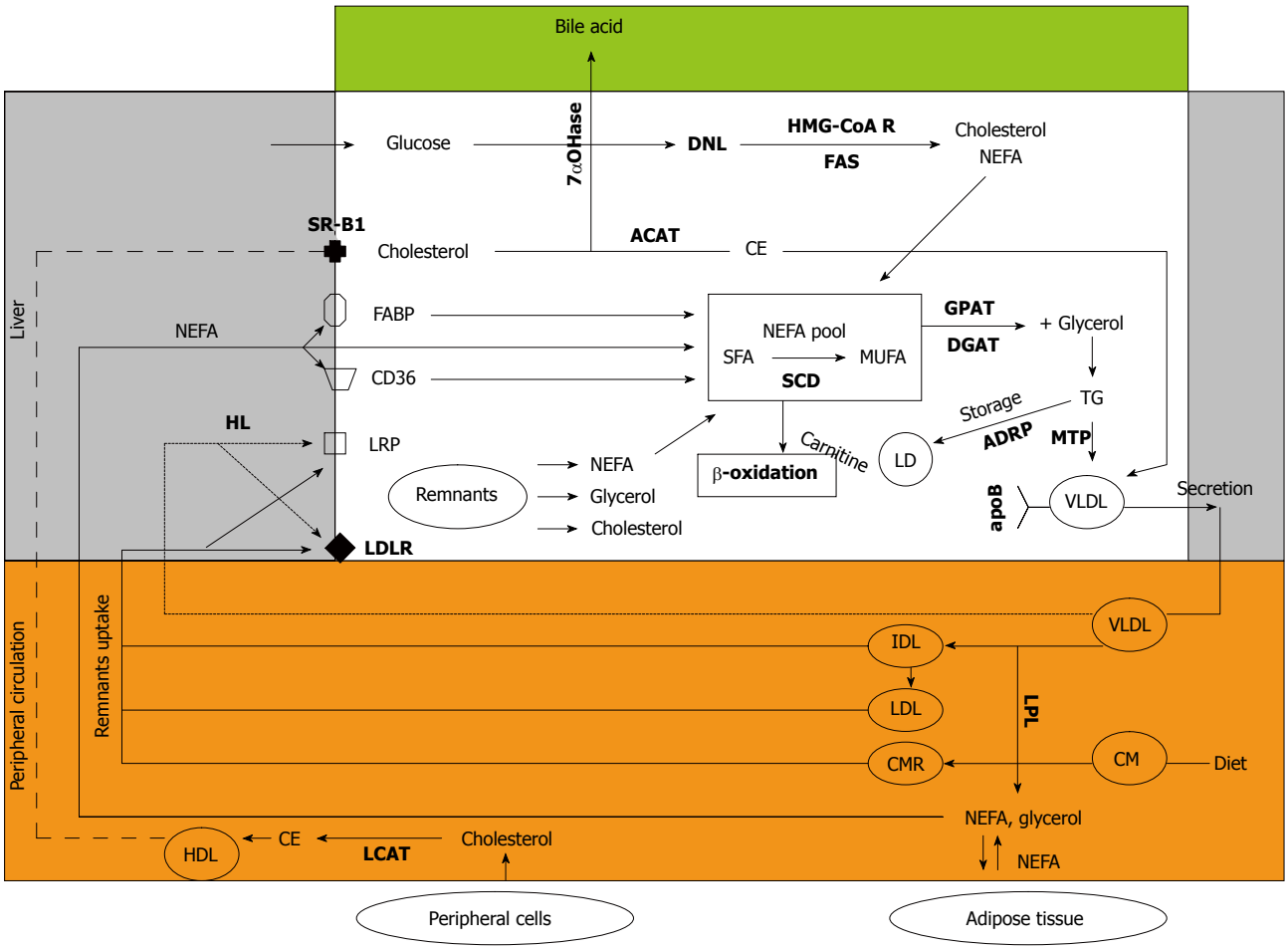


Figure 1 Possible sites (shown in bold) of interactions between iron and lipid metabolism. Iron deficiency can have its effects by generating anaemia/hypoxia while iron overload can interact by generating oxidative stress/lipid peroxidation and cytokine production. The grey area shows the basolateral surface of the hepatocyte bathed in the sinusoidal fluid. The green area is the canalicular surface through which bile is actively secreted into the biliary canaliculi. The orange area shows the lipoproteins and enzymes acting in peripheral circulation. CM: Chylomicron; CMR: Chylomicron remnant; VLDL: Very low density lipoprotein; IDL: Intermediate density lipoprotein; LDL: Low density lipoprotein; HDL: High density lipoprotein; LDLR: LDL (apo B/E) receptor; LRP: LDL receptor related protein; FABP: Fatty acid binding protein; FAT/CD36: Fatty acid translocase; SR-B1: Scavenger receptor-B1; NEFA: Non-esterified fatty acid; CE: Cholesteryl ester; SFA: Saturated fatty acid; MUFA: Mono-unsaturated fatty acid; TG: Triglycerides; apo-B: Apolipoprotein-B; DNL: *de novo* lipogenesis; LD: Lipid droplet; LPL: Lipoprotein lipase; HL: Hepatic lipase; LCAT: Lecithin cholesterol acyltransferase; GPAT: Glycerol-3-phosphate acyltransferase; DGAT: Diacylglycerol acyltransferase; SCD: Steroyl CoA desaturase; FAS: Fatty acid synthase; HMG CoA R: HMG CoA reductase; 7αOHase: 7α hydroxylase; ACAT: Acyl-CoA cholesterol acyltransferase; MTP: Mitochondrial Trifunctional protein; ADRP: Adipose differentiation-related protein.

lism appear to be related to effects of iron-deficiency on transcriptional/post-transcriptional mechanisms, as well as to effects on the kinetics/activity of enzymes that depend upon iron as a cofactor.

In contrast to the studies that show an increase in hepatic lipogenesis with iron deficiency, others have reported a decrease in hepatic lipid synthesis. In rats with moderate iron deficiency, glucose-fueled hepatic lipogenesis fell[27] which was attributed to decreased activity of either the rate limiting enzymes [fatty acid synthase (FAS)] or enzymes involved in providing the redox potential for lipogenesis (glucose 6 phosphate dehydrogenase, malic enzyme)[28-30].

In chicks, anemia depresses levels of mitochondrial cytochromes[31-33]. This finding is consistent with that found in yeast in which iron-deficiency down-regulates transcription of certain respiratory cytochromes that require heme as prosthetic groups for activity[34], while the heme degradation enzyme, heme oxygenase, is upregulated[35]. It is proposed that this decrease in the activity of hepatic heme pathways in iron-deficiency preserves the availability of iron for other essential sites/pathways when cellular iron levels are low[36]. Supporting this idea is the observation that when lipid metabolism is affected by iron deficiency in yeast, amino acid homeostasis remains unaltered[36].

Iron deficiency can affect the function of many hepatic enzymes involved in cholesterol metabolism[28]. In a mouse model of iron-deficiency induced by genetic mutation of hephaestin (sla mice), lack of the copper-dependent ferroxidase in the enterocyte results in impaired iron absorption and anaemia[37]. Under these conditions, hepatic cholesterol synthesis is reduced in association with decreased mevalonic acid levels. HMG CoA reductase activity, the rate limiting enzyme in *de novo* cholesterol synthesis, is increased in this model, arguing that this enzyme is not responsible for the decreased cholesterol production[37]. Similarly, acetyl-CoA thiolase and HMG-

CoA synthase involved in cholesterol production are not affected by iron deficiency[37]. All these enzymes act upstream in the production of mevalonic acid, suggesting that this intermediate is shunted to a non-steroidal pathway.

Other studies show that the cholesterol degradation pathway is also affected. One study in dogs shows that iron deficiency can decrease the activity of hepatic 7α hydroxylase, the rate limiting enzyme in the conversion of cholesterol into bile acids[38]. In this study, bile contained cholesterol crystals, suggesting that reduced conversion of cholesterol to bile acids may impair biliary micellar formation and explain the cholesterol precipitates in the bile. However, hepatic lipids were not measured in this study.

Nutritional iron depletion in rats also leads to alterations in the fatty acid composition of hepatic phospholipids, indicating impaired desaturation of saturated and essential fatty acids[29,39,40]. Steroyl CoA desaturase (SCD) is required for the conversion of saturated fatty acids to the unsaturated form and this enzyme is dependent on iron for its activity. Lower activity of SCD in iron deficiency may explain the increased saturated composition of fatty acids in the liver in this condition[29]. The effect of iron on the various steps in lipid metabolism is summarised in Figure 1.

It is likely that some of the reasons for the conflicting observations reported here as a result of iron deficiency can be explained in part by the specifics of the model system, by the means of inducing iron deficiency through phlebotomy or dietary restriction, or by the extent of the iron deficiency. Severe iron deficiency will produce anaemia and hypoxia which are likely to activate additional transcription factors to those stimulated during mild iron deficiency, and in turn, affect the transcription of more genes encoding enzymes/proteins[41]. Analysis of the reports cited in this review for correlations between the extent of iron deficiency and alterations in lipid metabolism was not possible, since the level of iron deficiency was not measured in these studies. It is recommended that future studies exploring effects of iron deficiency need to incorporate measurements of iron into their experimental design.

Another reason for the conflicting observations reported here might be the model systems themselves. Some animal model systems are designed to develop iron-deficiency from birth and others induce iron deficiency in a mature animal[4,23]. There are studies to suggest that metabolic conditions in development can impact on adult metabolism. For example, epidemiological data show that under normal birth weight conditions, there is an inverse correlation between the weight at birth and adult obesity[42,43]. Experimental data show that changing the diets during pregnancy or during the newborn period may permanently change the rate of lipid metabolism[44]. Therefore, it is important to recognize the possibility of fetal programming on hepatic lipid metabolism as an explanation for some of the differences reported.

EFFECTS OF IRON OVERLOAD ON HEPATIC LIPIDS

Iron overload can generate oxidative stress[45,46] and lipid peroxidation, which can modify the fatty acid profile of cellular membranes, leading to their disruption, damage to cell organelles[47-49] and impairment of mitochondrial oxidative metabolism[50,51]. It is suggested that the free radicals that form may cause a change in the ratio of saturated to unsaturated membrane phospholipids, leading to alterations in membrane fluidity[52]. This in turn may affect the activity of the embedded enzymes[53-56]. Enzymes respond to oxidative stress differently by altering their activity[55]. It is also known that the formation of peroxidation products by hepatocytes increases with the proportion of unsaturated fatty acids. Thus, polyunsaturated fatty acids in the presence of iron overload may exert an inhibitory effect on lipogenic genes (e.g., FAS) by generating a peroxidative cytotoxic effect[57] .

Iron overload also has direct effects on hepatic lipid metabolism, although studies report conflicting results based on different experimental models. Specific enzymes in hepatic cholesterol metabolism show variable responses to iron overload. In rats with dietary iron overload, an increase in the activity of acyl-CoA cholesterol acyltransferase (ACAT) coupled with reduced activities of HMG CoA reductase and 7 α-hydroxylase correlates with hypercholesterolemia and unaltered hepatic cholesterol content[55]. Since ACAT increases intrahepatic cholesterol esterification and contributes to VLDL-cholesterol secretion, these findings suggest that the cholesterol synthetic and excretory pathways are not affected while the secretory pathways maybe upregulated in this model system[55,58]. On the other hand, a recent study in mice by Graham *et al*[59], shows that transcripts of seven enzymes, including the rate limiting enzyme HMG CoA reductase, are up-regulated with increasing hepatic iron, suggesting that hepatic iron loading increases liver cholesterol synthesis. Although valuable, genomic analysis does not provide insight into enzyme kinetics and substrate concentration which may also be altered by the level of iron. This may explain some of the differences between these studies as well as different feeding regimes employed. Hepatic iron overload using carbonyl iron in the methionine-choline deficient rat model of NAFLD is associated with decreased hepatic TG and decreased hepatic steatosis; however, an improvement in liver injury is not seen due to increased necroinflammation and a trend towards increased perivenular fibrosis in iron loaded animals[58]. In carbonyl-iron and iron dextran models of iron overload in mice, studies show an upregulation of the mRNA and enzyme activity of SCD. SCD increases the biosynthesis of unsaturated fatty acids at 8 but not 2 mo of feeding[60]. In contrast, feeding ferric citrate for 3 mo has no effect on hepatic fatty acid composition or desaturase activity[40]. These findings suggest that the experimental model used, the level and duration of iron overload, as well as cellular repartition of iron storage in the liver may play a role in

SCD induction[60]. The effect of iron on the various steps in lipid metabolism is summarised in Figure 1.

In vitro studies in HepG2 cells show that iron overload increases intracellular lipid droplet formation by increasing the expression of cluster of differentiation 1d, an unconventional major histocompatibility complex class 1 molecule reported to monitor intracellular and plasma membrane lipid metabolism[61]. Both of these changes are also associated with increased expression of phosphatidylserine in the outer leaflet of the plasma membrane, a feature associated with apoptosis and cell death[62,63]. Hepatosteatosis has also been associated with an increased likelihood of apoptosis[64]. Cell death of mature hepatocytes disproportionate to their ability to regenerate has been referred to as a "third hit" in the pathogenesis of NAFLD/NASH leading to progression of fibrosis[65,66].

Several important factors may contribute to the inconsistencies described here in studies exploring effects of iron overload. These include differences in experimental designs, diets, age, sex, weight and strain of animals; differences in methods for generating iron overload; and also maternal status and its effect on fetal programming, as discussed for models of iron deficiency above.

ROLE OF IRON-OVERLOAD IN NON-ALCOHOLIC STEATOHEPATITIS

NAFLD is now accepted as an hepatic component of the metabolic syndrome[67]. It represents a spectrum of disease ranging from a benign state of non-alcoholic fatty liver to severe fibrosis or cirrhosis. NASH is the intermediate progressive stage between the two conditions. The exact mechanism for this progression is not known but a "two hit theory" is generally accepted. The first hit is considered to be insulin resistance, which leads to excessive accumulation of fat in the liver. The second hit is the generation of oxidative stress by a number of factors, including hepatic iron overload, which lead to cytotoxicity and necroinflammation. A number of clinical and animal studies have shown variable results concerning the effects of iron in NAFLD and NASH. This has lead to controversy about its role in the disease progression (Figure 2).

As already mentioned, excess iron is directly toxic to cells[47-49]. Furthermore, iron accumulation has a proinflammatory and profibrogenic role by activating Kupffer cells to release inflammatory cytokines[68,69] and by activating hepatic stellate cells, which can culminate in the replacement of parenchymal tissue with connective tissue[70]. Bacon *et al*[1], in 1994 first showed abnormal iron parameters (serum ferritin and transferrin saturation) and increased hepatic iron concentration in cases of NASH. Other clinical studies have also shown an increase of hepatic iron deposition in NASH/NAFLD and an increased prevalence of the hemochromatosis gene (*HFE*) mutation[2,3,71-73]. One study showed an association between increased hepatic iron and severity of fibrosis in NASH[2]. In contrast to the work of Bacon and others, however, there are also clinical studies that fail to find an increase in hepatic iron deposition in NASH[74-78] or an increase in HFE mutation[79-81], casting doubt on iron as a causative agent in the progression of the disease.

On the other hand, several clinical studies of NAFLD/NASH show an improvement in liver enzymes and insulin sensitivity with phlebotomies, even in cases without increased levels of iron[82-84]. One case report of a patient with NASH shows complete histologic resolution of steatohepatitis in the liver with phlebotomy-induced iron depletion[85]. However, it must be kept in mind that repeated phlebotomies might predispose to anemia, which could lead to tissue hypoxia and subsequent peripheral vasodilatation. Cardiac output would be expected to increase in response to reduced peripheral resistance, which in turn would increase tissue perfusion and peripheral lipoprotein clearance, reducing the lipid load on the liver. This proposed sequence of events is supported by hemodynamic studies in the anaemic state[86-88] and clearance studies using adrenergic agonists[89,90]. Taken together, an improvement in NAFLD/NASH observed with repeated phlebotomies might be more a physiological response to anaemia/tissue hypoxia rather than a direct effect of reducing iron toxicity. Care must be taken to choose this procedure in cardiac compromised patients.

It is a generally accepted hypothesis that iron overload may play a role in the progression of NAFLD *via* promoting the 2nd hit (Figure 2). However, it is also possible that the steatosis, cytokines and oxidative stress that are integral to NASH pathophysiology secondarily lead to iron overload, which then works in a feed-forward manner to generate more oxidative stress and inflammation, making the environment more favourable for the 3rd hit, injury that overwhelms the capacity of dying hepatocytes to regenerate and increases scar, as proposed by Diehl[65,91]. Supporting the idea, increased hepatic iron deposition is also seen in non-biliary causes of cirrhosis, such as alcoholic liver disease and chronic viral hepatitis as well as NASH[92]. The process of hepatocellular injury may itself result in increased iron uptake from the intestine[93]. The expression of transferrin receptor might be increased in regenerating hepatocytes after liver injury, which could contribute to increased hepatic iron loading[94]. Another mechanism of increased iron accumulation in liver in NAFLD and NASH might be due to changes in levels of ferroportin and the iron regulatory peptide hepcidin[95,96]. Rats developing insulin resistance on a high fat, high energy diet show decreased hepatic mRNA expression of hepcidin[97]. Similarly, mice developing steatosis on a choline deficient diet show a negative correlation of hepcidin and ferroportin (iron export molecule) mRNA with hepatic lipid concentration, suggesting that enhanced dietary intake and reduced hepatic iron efflux may lead to increased hepatic iron content[98]. In patients with NAFLD, there is also reduced expression of ferroportin and the iron-sensing molecule, hemojuvelin, which is associated with an increase of tumor necrosis factor-α, hepatic iron accumulation and increased hepcidin expression[99]. Hepcidin expression and iron regulatory molecules need to be further investigated in the livers of

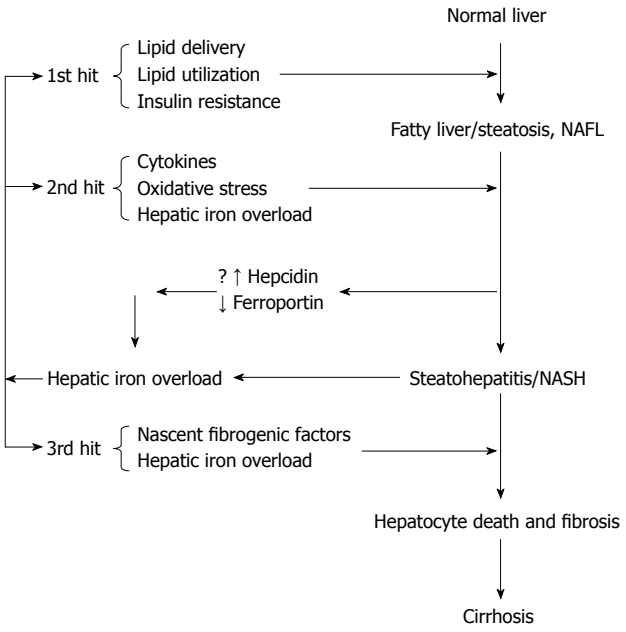


Figure 2 Schematic diagram depicting disease progression in non-alcoholic fatty liver disease modified after Gentile and Pagliassotti[101]. Iron overload is generally hypothesised to play a role in generation of second hit. In non-alcoholic fatty liver (NAFL) disease, iron overload in the liver may promote the development and progression of steatohepatitis, working in a feed-forward manner to promote the "third hit", on-going injury and death of hepatocytes leading to fibrosis and cirrhosis. NASH: Non-alcoholic steatohepatitis.

patients with metabolic syndrome and NAFLD.

It may also be the case that factors inducing localized iron deposition in the microenvironment of the liver are as, or more, important than total hepatic iron or systemic levels of iron. Iron in the microenvironment can promote regional oxidative stress that can lead to changes in microcirculation/capillarization, inflammation and fibrosis in NAFLD. In a rabbit model of steatohepatitis, Otogawa *et al*[100] have shown an increased phagocytosis of erythrocytes by Kupffer cells that is associated with increased hepatic iron deposition, suggesting that iron may accumulate locally as part of heme degradation. This finding is supported by a clinical study of patients with NASH in which aggregations of erythrocytes are seen in inflammed hepatic sinusoids[100].

In the aggregate, the findings reviewed here suggest the hypothesis that hepatic iron overload itself may be a critical event in the progression of NAFLD/NASH by exacerbating the 2nd hit of inflammation or perhaps by providing a critical 3rd hit that can ultimately lead to liver cell loss, failure of hepatocyte replication and eventual cirrhosis. Further studies are required to clearly establish the relationship between NASH/NAFLD, insulin resistance and iron overload.

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REVIEW

Diagnostic endoscopic ultrasonography: Assessment of safety and prevention of complications

Christian Jenssen, Maria Victoria Alvarez-Sánchez, Bertrand Napoléon, Siegbert Faiss

Christian Jenssen, Department of Internal Medicine, Krankenhaus Märkisch-Oderland GmbH, D15344 Strausberg, Germany
Maria Victoria Alvarez-Sánchez, Bertrand Napoléon, Department of Gastroenterology and Hepatology, Hopital Privé Jean Mermoz, F69008 Lyon, France
Siegbert Faiss, Department of Gastroenterology and Hepatology, Asklepios Klinik Barmbek, D22291 Hamburg, Germany
Author contributions: Jenssen C, Alvarez-Sánchez MV, Napoléon B and Faiss S contributed substantially to design and conception, performed the literature review and analyzed the literature data; Jenssen C provided the first draft of the paper, which was discussed and revised critically for intellectual content by Alvarez-Sánchez MV, Napoléon B and Faiss S; and all authors discussed the comments of the reviewers and approved the final version to be published.
Correspondence to: Christian Jenssen, MD, Department of Internal Medicine, Krankenhaus Märkisch-Oderland GmbH, Prötzeler Chaussee 5, D15344 Strausberg, Germany. c.jenssen@khmol.de
Telephone: +49-33-4152822 Fax: +49-33-45640302
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Abstract

Endoscopic ultrasonography (EUS) has gained wide acceptance as an important, minimally invasive diagnostic tool in gastroenterology, pulmonology, visceral surgery and oncology. This review focuses on data regarding risks and complications of non-interventional diagnostic EUS and EUS-guided fine-needle biopsy (EUS-FNB). Measures to improve the safety of EUS und EUS-FNB will be discussed. Due to the specific mechanical properties of echoendoscopes in EUS, there is a low but noteworthy risk of perforation. To minimize this risk, endoscopists should be familiar with the specific features of their equipment and their patients' specific anatomical situations (e.g., tumor stenosis, diverticula). Most diagnostic EUS complications occur during EUS-FNB. Pain, acute pancreatitis, infection and bleeding are the primary adverse effects, occurring in 1% to 2% of patients. Only a few cases of needle tract seeding and peritoneal dissemination have been reported. The mortality associated with EUS and EUS-FNB is 0.02%. The risks associated with EUS-FNB are affected by endoscopist experience and target lesion. EUS-FNB of cystic lesions is associated with an increased risk of infection and hemorrhage. Peri-interventional antibiotics are recommended to prevent cyst infection. Adequate education and training, as well consideration of contraindications, are essential to minimize the risks of EUS and EUS-FNB. Restricting EUS-FNB only to patients in whom the cytopathological results may be expected to change the course of management is the best way of reducing the number of complications.

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Key words: Endoscopic ultrasonography; Endoscopic ultrasonography-guided fine-needle biopsy; Complications; Contraindications; Risk; Safety; Perforation; Bleeding; Infection; Acute pancreatitis

Peer reviewers: Adrian Saftoiu, MD, PhD, Professor, Research Center of Gastroenterology and Hepatology Craiova, University of Medicine and Pharmacy Craiova, 2 Petru Rares str., 200349 Craiova, Romania; Dr. Massimo Raimondo, Division of Gastroenterology and Hepatology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, United States; Dr. Hyoung-Chul Oh, Assistant Professor, Department of Internal Medicine, College of Medicine, Chung-Ang University, No. 65-207 Hanganro-3ga Yongsan-gu, Seoul 140757, South Korea

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INTRODUCTION

Endoscopic ultrasonography (EUS) was first introduced

in 1980, when teams from the Wolfgang von Goethe University in Frankfurt, Germany[1] and the Mayo Clinic in Rochester, United States[2,3] incorporated a rotating mechanical ultrasound scanner or an electronic linear ultrasound array into the tip of side-viewing gastroscopes (Olympus GF-B3; ACMI FX-5), respectively. The clinical use of these early echoendoscopes was limited by the length (80 mm) and diameter (13 mm) of their rigid tips. Due to the devices' limited flexibility, endoscopists often were unable to pass the pyloric channel. Despite these disadvantageous mechanical properties, early reports showed no complications[1,3-5]. The first experience with needle biopsy under direct endosonographic guidance was published in 1992[6]. Over the last three decades, technological improvements have allowed EUS and EUS-guided fine-needle biopsy (EUS-FNB) to become established tools for the diagnosis, staging, and treatment of gastrointestinal diseases and benign and malignant pulmonary disorders[7-12]. In the United States, 60% of gastroenterologists use EUS, and approximately 43% of gastroenterologists and visceral surgeons in four European countries have access to EUS[13,14]. In Germany, EUS systems are used in universities and tertiary referral centers, as well as in 40% of the approximately 2000 hospitals[8]. Data from the prospective EUS registry of the German Society of Ultrasound in Medicine show that EUS-FNB is performed during one out of seven endosonographic examinations. The two basic EUS-FNB techniques are: EUS-guided fine-needle aspiration (EUS-FNA) using hollow-bore 19 Gauge (G) to 25 G needles, and EUS-guided trucut-biopsy (EUS-TCB) using side-notch 19 G core needles[8]. After 30 years of clinical experience with EUS and 20 years' experience with EUS-FNB, reliable data on the incidence of complications and specific risk factors are available and may be used as the background information when obtaining informed consent.

EUS-SPECIFIC RISK FACTORS

Endoscopic ultrasound systems differ from traditional forward-viewing and oblique-viewing endoscopes in the rigidity and stiffness of the scope tip that carries the ultrasound transducer. Moreover, echoendoscopes have a somewhat larger diameter (12.4 mm to 14.6 mm) compared to gastroscopes (9.0 mm to 9.8 mm) and duodenoscopes (11.0 mm to 13.1 mm), and a less flexible bending section[15]. Additionally, most echoendoscopes have a longer non-flexible segment just proximal to the transducer (Figure 1). The mean EUS examination time (20 min in a German multicenter survey[16] or 34 ± 20 min in one US-center with a high case load[17]) is significantly longer than that needed for standard esophagogastroduodenoscopy. With the exception of the electronic radial scanning echoendoscopes of Pentax/Hitachi and Fujinon, all other currently available echoendoscopes are oblique-viewing instruments[15]. Therefore, intubation and advancement of the echoendoscopes, especially through the esophagus, are semi-blind maneuvers. Moreover, in oblique-viewing instruments, the rigid segment containing the transducer extends beyond the optical lens.

EUS-FNA and EUS-TCB differ from forceps biopsies in that the needle penetrates from the non-sterile gastrointestinal tract into sterile extra-intestinal anatomical structures to access the target organ or structure. The target lesions are often near to vascular structures[18] and may themselves have pathological vascularization. For these reasons, it is not possible to apply safety data directly from upper gastrointestinal endoscopy to upper gastrointestinal EUS.

COMPLICATIONS OF NON-INTERVENTIONAL DIAGNOSTIC EUS

Gastrointestinal perforation

Due to its mechanical and optical properties, echoendoscope advancement may perforate the gastrointestinal wall, particularly at: (1) areas of angulation (e.g., hypopharynx, hiatal hernia, tip of the duodenal bulb, and rectosigmoidal junction); (2) in the presence of unexpected anatomical alterations (e.g., esophageal or duodenal diverticula); and (3) in luminal obstruction (e.g., gastrointestinal cancer). Several investigations of EUS staging have documented non-transversable malignant stenosis in 15% to 42% of patients with esophageal cancer[19-25], and in 7% to 23% (mean 12%) of patients with rectal cancer[26].

Esophageal perforation: In a survey of members of the American Endosonography Club published in 2002, 86 members reported 16 cervical esophageal perforations among 43 852 upper gastrointestinal EUS procedures performed using radial echoendoscopes (0.03%)[27]. Almost all of these patients were elderly; seven of the 16 (44%) had a history of difficult intubation during previous endoscopy. Large cervical osteophytes were identified in three cases. In nine of 16 cases (56%), the endoscopist had less than one year of experience in EUS. Two patients required surgery. For 13 patients (81%), the complication was managed successfully with conservative treatment. However, one patient died[27].

Another early multicenter survey including 34 centers from Europe, Japan and the United States[28] reported 13 esophageal perforations in 37 915 radial EUS examinations (0.03%); esophageal perforations accounted for 68% of all complications. Most of these patients (77%) had undergone pre-EUS dilatation of esophageal strictures to allow complete esophageal cancer staging, including celiac lymph-node station. Nine of these patients underwent surgery, four were managed conservatively, and one patient died. Further major complications in this survey were two pharyngeal and one duodenal perforation and two cases of bleeding[28].

Only two studies have investigated the frequency of esophageal perforations prospectively. A single-center study in the United States enrolled all patients who underwent upper gastrointestinal EUS by the same experienced endosonographer over a 7-year period. Cervical esophageal perforations occurred in three of 4844 pa-

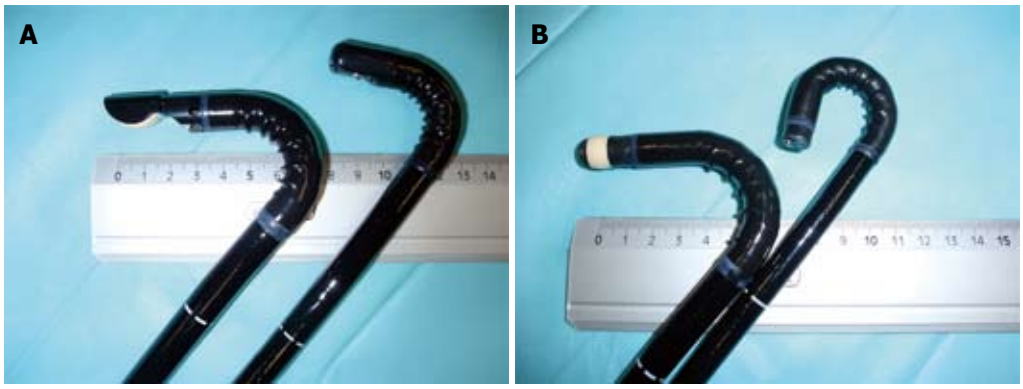


Figure 1 Flexibility and rigid tip of echoendoscopes and standard videoendoscopes. A: Longitudinal video-echoendoscope EG-3870 UTK (left; Pentax Medical/Hitachi Medical Systems; angulation up/down 130/130 degrees) with an approximately 50-mm long rigid tip at the distal end (diameter 12.8 mm). The therapeutic video-duodenoscope ED-3490 TK from the same manufacturer (right; Pentax Medical; distal outer diameter 11.6 mm, angulation up/down 120/90 degrees); B: Radial video-echoendoscope EG-3670 URK (left; Pentax Medical/ Hitachi Medical Systems; angulation up/down 130/60 degrees) with an approximately 50-mm long rigid tip at the distal end (diameter 12.1 mm) and the standard video-gastroscope EG-2990i from the same manufacturer (right: Pentax Medical; distal outer diameter 9.8 mm, angulation up/down 210/120 degrees).

Table 1 Frequency of upper gastrointestinal tract perforations caused by radial and longitudinal echoendoscopes in the German survey of endoscopic ultrasonography complications (2004-2006, data from Jenssen, Faiss and Nürnberg[31]) *n* (%)

Perforation location	Radial EUS (*n* = 47 417)	Longitudinal EUS (*n* = 37 667)	Radial and longitudinal EUS[1] (*n* = 85 084)
Esophageal	3 (0.087)	5 (0.013)	8 (0.009)
Duodenal	8 (0.017)	11 (0.029)	19 (0.022)[a]
Gastric	0	1 (0.003)	1 (0.001)
Total	11 (0.023)	17 (0.045)	28 (0.033)

[1]Without endoscopic ultrasonography (EUS)-guided fine-needle biopsy or EUS-guided therapeutic interventions. [a]$P < 0.05$ *vs* esophageal perforations.

tients (0.03%). Longitudinal echoendoscopes had been used in all three patients[29]. The second prospective study, performed in a department of surgical endoscopy in Denmark, evaluated EUS complications of 3324 consecutive patients. Esophageal perforation occurred in 5 of 10 patients with complications (0.15% of all EUS examinations; 0.94% of all patients with esophageal cancer). Balloon dilation had been performed prior to EUS in two cases. All five patients recovered fully with conservative (*n* = 4) or surgical treatment (*n* = 1)[30].

From 2004 to 2006, we conducted a survey in German centers performing EUS[31]. Of 67 centers responding to the questionnaire, 32 registered EUS complications prospectively. Esophageal perforation occurred in only eight of 85 084 reported diagnostic EUS procedures (0.009%). None of the perforations were associated with previous dilation of esophageal strictures. Stenosing esophageal cancer was present in five of eight cases[31].

Duodenal perforations: In contrast to the international multicenter survey conducted from 1982 to 1992[28], the German retrospective survey showed that duodenal perforations occurred significantly more often (19 additional cases) compared to esophageal perforations (0.022%)[31]. In 10 of 19 cases (47.4%), duodenal diverticula (*n* = 4), duodenal stenosis (*n* = 3), duodenal ulcer (*n* = 1), duodenal scarring (*n* = 1), or acute pancreatitis (*n* = 1) were reported as potentially contributing factors. Twenty-seven of 28 gastrointestinal perforations were managed surgically, and all the patients survived[31] (Table 1). In a prospective EUS online registry of the German Society of Ultrasound in Medicine, participants reported 10 cases of gastrointestinal perforation in 13 988 diagnostic EUS procedures (0.07%). Again, duodenal perforation was the most common type of perforation, accounting for six out of ten cases[32]. The increasing proportion of duodenal perforations in recent multicenter studies compared to the older surveys may partially reflect changing trends in indications for EUS[7,33].

In a large series of 233 EUS-FNA biopsies in patients with presumed pancreatic cancer, Raut and colleagues reported two cases of duodenal perforation requiring surgical intervention (0.86%)[34]. There was no luminal narrowing of the duodenum in either case[34]. One published case report describes iatrogenic duodenal perforation during EUS, which was managed successfully by endoscopic closure using hemoclips, followed by conservative treatment[35]. In a series of 224 EUS-FNAs, one duodenal perforation accounted for one of five severe complications[36]. A large single-center series of 1034 pancreatic EUS-FNAs found one case of fatal duodenal perforation in a 63-year-old woman with an advanced neuroendocrine tumor of the duodenal wall; the perforation likely resulted from mechanical injury of the duodenal wall by the echoendoscope, rather than from biopsy[37].

A national survey in Israel which investigated mortality associated with diagnostic EUS[38] showed that 13 of 18 reported fatal complications (seven in Israel and six from outside the country) resulted from duodenal tears which led to retroperitoneal perforations. Two of the fatalities were secondary to esophageal perforation. At least four of six cases of duodenal perforation reported from Israel involved patients with duodenal diverticula. Five of

Table 2 Possible risk factors for endoscopic ultrasonography-related gastrointestinal perforations (adapted from Jenssen, Mayr, Nuernberg and Faiss[40])

Risk factor	References
Operator inexperience	Das *et al*[27], Jenssen *et al*[31], Lachter[38]
Esophagus	
Stenosing cancer of the esophagus and esophagogastric junction	Das *et al*[27], Mortensen *et al*[30], Rösch *et al*[28]
Dilatation of esophageal stricture prior to EUS	Das *et al*[27], Mortensen *et al*[30], Rösch *et al*[28]
Advanced patient age	Das *et al*[27]
Difficult previous endoscopic esophageal intubation	Das *et al*[27]
Large cervical osteophytes	Das *et al*[27]
Duodenum	
Diverticula	Jenssen *et al*[31], Lachter[38]
Stenosis, pancreatic head tumor	Raut *et al*[34]
Longitudinal echoendoscope (long, rigid tip)	Lachter[38]

EUS: Endoscopic ultrasonography.

eight fatal complications in Israel occurred during examinations by endoscopists who had performed fewer than 300 EUS procedures[38].

Other gastrointestinal perforations: EUS-related gastric and rectal perforation seems very rare. There is only one case of rectal perforation reported in the prospective German EUS registry[32]. One study of 2490 endorectal ultrasound examinations reported no procedure-related perforations[39]. One case of gastric perforation occurred in each of the German retrospective study[31] and the German prospective registry[32]. In all three cases of rectal or gastric perforations the indication for EUS was staging of a stenosing tumor[31,32]. A few pharyngeal and hypopharyngeal perforations have been reported as well[28,32]. Possible risk factors for EUS-related gastrointestinal perforations are summarized in Table 2.

Aspiration

For appropriate acoustic coupling of the scanner to the gastrointestinal wall, EUS textbooks suggest instillation of water into the gastric lumen for some indications (e.g., gastrointestinal cancer staging, examination of subepithelial tumors). In a Danish prospective study, tracheal suction was needed in 15 of 293 patients (5.1%), and one patient aspirated during the procedure (0.3%). However, the "water-in-stomach" method was only used in nine patients (3.1%)[30]. Only five cases of aspiration pneumonia have been reported following EUS[32,38,41].

Bacteremia

Few data are available concerning the frequency of bacteremia following diagnostic EUS without EUS-FNB. Janssen *et al*[42] investigated the incidence of bacteremia in 100 consecutive patients undergoing longitudinal diagnostic EUS using paired blood cultures: immediately before and again 5 min after EUS. Significant bacteremia (*Streptococcus viridans* and *Propionibacterium* species; *Streptococcus mitis*) occurred in only two patients (2%), both of whom underwent endosonographic staging of esophageal cancer (seven of nine patients with esophageal cancer did not experience bacteremia)[42]. Levy *et al*[43] noted transient bacteremia in one of 52 patients who underwent diagnostic EUS with a radial-scanning echoendoscope (1.9%). None of these three patients with bacteremia following diagnostic non-interventional EUS in the two studies developed symptoms of infection[42,43].

Hematogenous tumor cell dissemination

Hematogenous tumor cell dissemination has been documented in 11 of 45 patients (24%) following transrectal ultrasound (TRUS)-staging of rectal cancer. In 17 other patients (38%), circulating tumor cells were detected in peripheral blood samples before and after TRUS[44]. The clinical and prognostic significance of this observation is presently unclear, although data from the same group suggest that the detection of circulating tumor cells in blood samples of patients with stage Ⅱ colorectal cancer correlates with poor outcome[44]. However, a recent retrospective analysis of metastatic rates of locally advanced esophageal cancer showed no difference between those who required and those who did not require esophageal dilation to complete EUS staging[25]. Prospective data are needed to determine whether passage of an endoscope beyond a stenosing gastrointestinal tumor causes clinically relevant hematogenous tumor cell dissemination.

The influence of age on EUS complications

EUS is reported to be safe in pediatric and elderly populations. No complications were reported in five case series of EUS and EUS-guided procedures performed in a total of 166 children[45-49] plus several case reports. A retrospective analysis of EUS and EUS-FNB in 265 consecutive patients aged 80 years or older showed that diagnostic EUS is feasible and safe in geriatric patients[50]. Another study compared complications of endoscopic retrograde cholangiopancreatography (ERCP) and EUS in elderly (≥ 75 years, n = 184) and non-elderly (< 75 years, n = 816) patient cohorts. The EUS complication rate of 4.8% among elderly patients did not statistically differ from the 3.1% complication rate among non-elderly patients[51].

Table 3 Complications of diagnostic endoscopic ultrasonography in 67 German centers (2004-2006, data from Jenssen, Faiss and Nürnberg[31]) *n* (%)

Complications	Non-interventional diagnostic EUS (*n* = 85 084)	EUS-FNB (*n* = 13 223)	Total (*n* = 98 307)
Total	29 (0.034)	38 (0.29)	104 (0.10)
Major	29 (0.034)	23 (0.17)	52 (0.053)
Conservative treatment	2	15	17
Interventional treatment	0	4	4
Surgical treatment	27	4	31
Mortality	4 (0.005)	0 (0.000)	4 (0.004)
Minor (no treatment necessary)	0	15 (0.11)	16 (0.016)

EUS: Endoscopic ultrasonography; EUS-FNB: Endoscopic ultrasonography-guided fine-needle biopsy.

Table 4 Procedural risk of endoscopic ultrasonography

First author (yr)	Study features	Frequency of complications (%)		
		EUS[1]	EUS-FNB	EUS-I
Prospective, single-center				
Williams *et al*[52]	333 EUS-FNA	-	0.3	-
Mortensen *et al*[30]	2518 EUS; 670 EUS-FNA; 136 EUS-I	0.22	0.3	0.74
Bournet *et al*[36]	3207 EUS; 224 EUS-FNA	0.093	2.2	-
Eloubeidi *et al*[53]	355 pancreatic EUS-FNA	-	2.54[2]	-
Al-Haddad *et al*[54]	483 EUS-FNA	-	1.4	-
Eloubeidi *et al*[55]	656 EUS-FNA	-	1.1[2]	-
Thomas *et al*[56]	247 EUS-TCB	-	2.4[3]	-
Gerke *et al*[57]	44 EUS-TCB; 36 EUS-FNA[4]	-	2.3 (EUS-TCB); 2.8 (EUS-FNA)	-
Prospective, multicenter (registry)				
Gottschalk *et al*[32]	11 889 EUS; 2099 EUS-FNB; 438 EUS-I	0.14	2.1	4.1
Retrospective, single-center				
O'Toole *et al*[41]	322 EUS-FNA	-	1.6	-
Carrara *et al*[37]	1034 pancreatic EUS-FNA	-	1.25	-
Retrospective, multicenter				
Rösch *et al*[28]	42 105 EUS (34 EUS centers)	0.05	-	-
Wiersema *et al*[58]	457 EUS-FNA (4 EUS centers)	-	1.1	-
Buscarini *et al*[59]	11 539 EUS; 787 EUS-FNA; 21 EUS-I (6 EUS centers)	0.046	0.88	9.5
Jenssen *et al*[31]	85 084 EUS; 13 223 EUS-FNB; 2297 EUS-I (67 EUS centers)	0.034	0.29	1.61

Results of large single-center and multicenter studies on complications of non-interventional diagnostic endoscopic ultrasonography (EUS), endoscopic ultrasonography-guided fine-needle biopsy (EUS-FNB) [endoscopic ultrasonography-guided fine needle aspiration (EUS-FNA) plus endoscopic ultrasonography-guided trucut biopsy (EUS-TCB)] and endoscopic ultrasonography-guided therapeutic interventions (EUS-I). [1]Without EUS-guided fine-needle biopsies and EUS-guided therapeutic interventions; [2]major complications only; 1 fatality due to pulmonary embolism; [3]EUS-guided trucut-biopsy (19 Gauge); [4]randomized controlled trial.

COMPLICATIONS OF EUS-FNB

Patients who undergo EUS-FNB are approximately ten times more likely to suffer complications or report symptoms following EUS compared to patients undergoing diagnostic non-interventional EUS (Tables 3 and 4)[30-32,36,59,60].

According to a recent systematic review of 51 EUS-FNB trials, the most common complications are post-procedural pain (34%), acute pancreatitis (34%), fever and infectious complications (16%), and bleeding (13%). Perforations and bile leaks are uncommon (3%). The overall complication rate of EUS-FNB in this systematic analysis of 10 941 patients was 0.98% (1.72% in 31 prospective studies). The procedure-related mortality was estimated at 0.02%[61].

Bacteremia and infectious complications

Bacteremia following upper gastrointestinal tract EUS-FNB appears to be rare. Its frequency has been investigated in three prospective studies of 202 patients[42,43,62]. In one study of 100 patients, all blood cultures obtained 30 min and 60 min after the procedure were negative, except in six patients in whom the culture was positive for coagulase-negative *Staphylococcus*, which the investigators regarded as contaminating and non-pathogenic[62]. Among 102 patients studied by Janssen *et al*[42] and Levy *et al*[43], five had bacteremia following EUS-FNB [three with *Streptococcus viridans*, one with *Streptococcus* group F (1), and one with *Gram-negative bacilli*]. No signs or symptoms of infection developed in these five patients. Large case series of EUS-FNB for solid pancreatic mass showed that 0.4% to 1.0% of patients experienced febrile episodes following the procedure[63,64]. In one patient undergoing EUS-FNA of a large pancreatic head mass, pyrexia and abdominal pain emerged followed by extensive acute portal vein thrombosis[65]. In addition to two cases of

fever after lymph node EUS-FNA which resolved with antibiotics[54,66], there have been 12 published reports of serious infectious complications following EUS-FNA or EUS-TCB of mediastinal lymph nodes. In one case, a mediastinal abscess developed after EUS-guided aspiration biopsy of a mediastinal lymph-node metastasis from hepatocellular carcinoma (HCC). The iatrogenic abscess was treated successfully by EUS-guided drainage and subsequent endoscopic closure of the resulting esophagomediastinal fistula[67]. Another mediastinal abscess developed following EUS-FNA of a mediastinal metastasis of malignant teratoma; the lesion required computed tomography (CT)-assisted drainage and subsequent thoracotomy[68]. Infectious mediastinitis has also occurred as a result of transesophageal EUS-FNA of an enlarged necrotic lymph node[69] and of a small benign lymph node in the aortopulmonary window[70]. Another case of mediastinitis after EUS-FNB for mediastinal lymphadenopathy developed as a consequence of perforation due to technical problems with the needle[71]. In one patient, EUS-FNA of a tuberculous subcarinal lymph node led to multiple symptomatic mediastinal-esophageal fistulae, which resolved in the course of tuberculostatic treatment[72]. Only one case has been published describing infectious endocarditis (*Streptococcus salivarius*) secondary to EUS-FNA of a mediastinal lymph node in an oncology patient[73]. Recently, five cases of mediastinal abscess have been reported following EUS-FNA in patients with sarcoidosis; four required surgical treatment[74].

There are six published reports of infection after EUS-FNA or EUS-TCB of subepithelial tumors. In one case, EUS-FNA of an esophageal leiomyoma caused severe intramural infection which resulted in esophagectomy[75]. In another case, fever developed 2 h after EUS-FNA of a large mesenchymal esophageal tumor, presumably due to left lobar pneumonia[41]. In the third case a large duodenal gastrointestinal stromal tumor (GIST) became infected with *Enterobacter cloacae* following EUS-FNA. The resulting abscess resolved following endoscopic transduodenal drainage and antibiotic treatment[76]. In a series of 52 consecutive EUS-TCBs of gastric subepithelial tumors, two cases of severe septic complications (*Streptococcus* sepsis and gastric wall abscess) occurred (3.9%)[77]. In our own series of 46 EUS-guided biopsies of hypoechoic subepithelial gastric tumors using a 19 G aspiration-needle, one extramural abscess and septic infection (*Morganella morganii*) developed in a patient with a large gastric GIST who was receiving 15 mg of prednisolone per day[78]. Despite the high colonization rate of the lower digestive tract lumen with pathogenic bacteria, EUS-FNB of (peri-)rectal and (peri-)colonic solid masses appears relatively safe. Only two cases of infectious complication following transrectal or transcolonic EUS-FNB of solid mass lesions has been published[79,80]. In a prospective risk assessment of bacteremia in 100 patients undergoing EUS-FNB of rectal and perirectal lesions, six patients developed positive blood cultures[81]. However, four of these cases were considered to be contaminants (coagulase-negative *Staphylococcus*, *Moraxella*, *Peptostreptococcus stomatis*). Two patients had true-positive blood cultures (*Bacteroides fragilis* and *Gemella morbillorum*). No immediate or delayed infectious complications occurred in any patient[81]. Five other studies (476 patients) showed no complications in patients who underwent transrectal or transcolonic EUS-FNB of solid masses[82-85].

In contrast, EUS-FNB of cystic lesions appears to carry a significant risk of severe infectious complications. In a large multicenter study of EUS-FNA, two of 18 patients undergoing EUS-FNA for cystic pancreatic lesions without peri-procedural antibiotics developed cyst infection[58]. Despite the large number of EUS-guided diagnostic procedures performed for cystic pancreatic lesions, there have been only four reports of infection after EUS-FNB performed with prophylactic antibiotics[52,86,87]. Of the 909 patients who underwent EUS-FNB of pancreatic cystic lesions included in the systematic review of Wang *et al*[61], 93.7% received prophylactic antibiotics (as recommended by the American Society of Gastrointestinal Endoscopy[88]), and the rate of cyst infection was estimated at 0.55%. However, there is no randomized prospective study supporting the utility of peri-procedural antimicrobial therapy. One recent retrospective study comparing the incidence of infectious complications after 263 EUS-FNBs of cystic pancreatic lesions for 88 cases with and 178 cases without antibiotic prophylaxis showed no significant difference[86]. The authors of one experimental study proposed to implement the combination of systemic antibiotics and local 5% povidone iodine during EUS-FNB of cystic lesions[89].

EUS-FNB of bronchogenic and other mediastinal cysts may cause cyst infection, mediastinitis and severe sepsis. Foregut duplication cysts account for 6% to 15% of primary mediastinal masses[90]. The differentiation between esophageal bronchogenic cysts and subepithelial tumors or paraesophageal masses may be difficult because of the hypoechoic and inhomogeneous mucoid content[91,92]. One study of 19 patients with bronchogenic cysts diagnosed by EUS demonstrated that 13 were anechoic, but the other six were hypoechoic, which suggested, erroneously in these cases, a solid mass lesion. CT or magnetic resonance imaging were diagnostic for a cyst in only four cases[93]. In another study, 9 of 27 benign mediastinal cysts were misdiagnosed as solid masses by CT[94]. Eight cases have been reported of mediastinitis following EUS-FNA or EUS-TCB of mediastinal mass lesions that were hypoechoic on EUS and had high CT attenuation values, but were later shown to be bronchogenic cysts. Because solid lesions were suspected, 5 of these patients did not receive antibiotics[36,92,93,95,96]. In three other cases, severe infection requiring surgical intervention occurred despite pre- and post-interventional intravenous antibiotics[97]. Moreover, one case of asymptomatic contamination of a mediastinal foregut duplication cyst with *Candida albicans* developed following pre-procedural antibiotic prophylaxis[98]. However, no infectious complications were seen among 22 patients who underwent transesophageal EUS-FNA of suspected mediastinal cysts using peri-interventional antibiotic prophylaxis[91].

In one recently published study on the utility of EUS-guided biopsy of extramural pelvic masses, two patients with cystic pelvic masses developed abscesses (7%)[99]. Also EUS-FNA of ascites and pleural fluid carries a risk of infection. Despite levofloxacin prophylaxis, one of 25 patients undergoing transgastric or transduodenal EUS-FNA of suspected malignant ascites developed bacterial peritonitis. Treatment with intravenous antibiotics was successful[100]. In another study, two of 60 patients (3%) developed self-limited fever following EUS-guided paracentesis[101]. Three other studies (47 patients) did not report any complications following EUS-FNA of ascites or peritoneal nodules in ascites in patients who did not receive prophylactic antibiotics[102-104].

Biliary peritonitis and cholangitis

Biliary peritonitis is a severe complication which may develop secondary to penetration or perforation of the common bile duct during EUS-FNB of a pancreatic mass in patients with biliary obstruction[105]. During a study to identify patients with microlithiasis as a cause of idiopathic pancreatitis, EUS-guided gallbladder bile duct aspiration resulted in biliary peritonitis in two of the three patients enrolled; these complications prompted the investigators to discontinue the study[106]. However, a recent systematic review of nine studies (284 patients) showed EUS-FNB of gallbladder masses or bile duct strictures to be safe, with no reported complications[107]. During an international survey of the indications, yield, and safety of EUS-FNB of liver lesions, one death due to septic cholangitis associated with EUS-FNB of a liver metastasis was reported. The patient who died had an occluded biliary stent, with biliary obstruction secondary to pancreatic cancer[108]. Mechanical irritation caused by the tip of the scope or by a water-filled balloon during pancreatic EUS may cause proximal or distal biliary stent migration, possibly followed by cholangitis[109].

Acute pancreatitis

Hyperamylasemia was common following pancreatic EUS-FNB in one recent prospective study, occurring in 11 of 100 patients. However, only two patients developed mild acute pancreatitis[110]. Nine large studies have reported iatrogenic acute pancreatitis following EUS-FNB of pancreatic lesions, with rates ranging from 0.19% to 2.0%[37,41,52,53,60,111-113]. A pooled analysis of 4909 solid pancreatic mass EUS-FNBs from 19 US centers identified 14 cases of iatrogenic post-procedural acute pancreatitis (0.29%)[114]. Among them, 71% were mild, 21% moderate, and 7% severe. One patient with significant co-morbidity died secondary to severe procedure-related pancreatitis. The frequency of acute pancreatitis at individual centers ranged from 0% to 2.35%. Only two centers (413 cases of pancreatic EUS-FNB, two cases of acute pancreatitis) assessed EUS-FNB complications prospectively. Some centers with retrospective complication assessment reported no cases of iatrogenic acute pancreatitis, suggesting that acute pancreatitis may have been underreported for the retrospective cohort. Interestingly, the survey showed that half of patients who developed acute pancreatitis after EUS-FNB had benign disease, suggesting that these patients may carry a higher risk. Another possible risk factor for iatrogenic acute pancreatitis following EUS-FNB is a history of acute recurrent pancreatitis[114]. In a German retrospective survey, eight of nine patients (86%) with reported acute pancreatitis after pancreatic mass EUS-FNB had benign disease[31]. In a large retrospective series of EUS-FNB for pancreatic cystic lesions, acute pancreatitis occurred in six of 603 cases (1%)[52]. A retrospective analysis of pancreatic EUS-FNB detected pancreatitis in three of 114 (2.6%) patients with cystic lesions, but none in the 134 patients with solid lesions[41].

Two studies have investigated the value of EUS-FNA and EUS-TCB in the diagnosis of chronic pancreatitis. Mild acute pancreatitis developed in two of 27 patients (7.4%) following EUS-FNA[115] and in one of 16 patients (6%) following EUS-TCB[116].

Pancreatic leak

This rare complication was reported only recently in two cases following EUS-FNA of pancreatic mass lesions. Both cases resolved after antibiotic treatment, transpapillary duct drainage and percutaneous drainage[117,118].

Hemorrhage

Self-limiting mild intraluminal bleeding due to EUS-FNB has been reported in up to 4% of cases[64] (Figure 2). A prospective study evaluating the risk of extraluminal hemorrhage caused by EUS-FNA found only three cases among 227 patients (1.3%)[119]. Extraluminal bleeding following EUS-FNB can be visualized clearly using EUS. Bleeding appears as an expanding hyperechoic or hypoechoic region adjacent to the sampled lesion[119]. Occasionally, color-coded Doppler may demonstrate blood flow within a "patent needle track" (Figure 3). In a retrospective survey of German EUS centers (13 223 EUS-FNB; Table 3), bleeding was the most common complication of EUS-FNB (0.15% of cases)[31]. Mild and self-limited bleeding was reported in 15 patients, whereas five patients suffered from severe hemorrhages requiring transfusion, and two of those patients required surgical intervention. One patient was treated with low-molecular-weight heparin (LMWH) immediately after the procedure and developed a large mediastinal hematoma and hemothorax; thoracoscopy and transfusion of seven units of packed red cells were required. Another patient required transfusion of four units of packed red cells following EUS-FNB of pancreatic head carcinoma with associated portal hypertension[31]. The prospective German EUS registry also showed that bleeding was the most frequent complication, reported in 1.4% of cases of EUS-FNB. However, 29 of 30 cases were judged to be mild, requiring no endoscopic intervention, surgery, or transfusion[32]. Patients with highly vascularized lesions (e.g., subepithelial mesenchymal tumors, neuroendocrine tumors, and some metastases), as well as those with cystic lesions, may be at greater risk. An Ital-

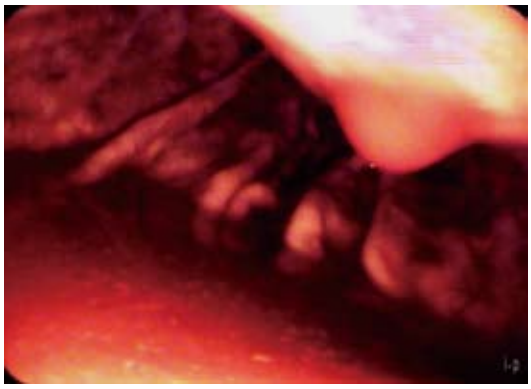


Figure 2 Intraluminal bleeding after endoscopic ultrasonography-guided fine-needle aspiration. Marked intraluminal bleeding occurred following 19 G endoscopic ultrasonography-guided fine-needle aspiration of a large, hypervascularized gastrointestinal stromal tumor in the stomach. Hemorrhage stopped without intervention.

ian multicenter retrospective study of EUS complications showed that three out of seven EUS-FNA complications were intracystic bleeding[59]. Three of 50 patients (6%) in a retrospective study developed acute intracystic hemorrhage following EUS-FNB of pancreatic cystic lesions[120]. Two patients experienced transient abdominal pain, and none of the three patients required blood transfusion. Intracystic bleeding episodes were readily recognized during the investigation as expanding hyperechoic areas surrounding needle puncture sites within the target cysts. Contributing risk factors could not be identified for the bleeding events[120]. In a large Italian single-center experience, bleeding was the most frequent pancreatic EUS-FNA complication (0.96%; n = 1034), with seven of ten cases occurring in cystic lesions[37].

A prospective analysis compared the risk of bleeding after EUS-FNA or EUS-TCB in patients taking non-steroidal anti-inflammatory drugs (NSAIDs), aspirin (ASA) or prophylactic LMWH to those who did not receive those medications[121]. Two of six patients (33.3%) receiving prophylactic LMWH developed extraluminal bleeding, compared to 0 of 26 patients receiving platelet inhibitors, and 7 of 190 patients (3.7%) not receiving those medications. There appears to be no increased bleeding risk during EUS-FNB for patients taking ASA or NSAIDs, but a possible risk for patients receiving prophylactic LMWH[121]. There are no published data regarding the risk of EUS-FNB in patients treated with clopidogrel and other thienopyridines. However, one study of patients undergoing transbronchial lung biopsy showed that clopidogrel, especially when combined with ASA, greatly increased bleeding risk[122].

There have been several case reports of severe bleeding associated with EUS-FNB, including hemosuccus pancreaticus in cystic pancreatic lesions[123], left adrenal gland hemorrhage[124], retroperitoneal bleeding following EUS-FNA of solid and cystic pancreatic lesions[59,125,126], mesenteric bleeding[127], and intramural bleeding following EUS-TCB of subepithelial tumors[78,96,128]. Three fatal cases of extraluminal bleeding have been reported

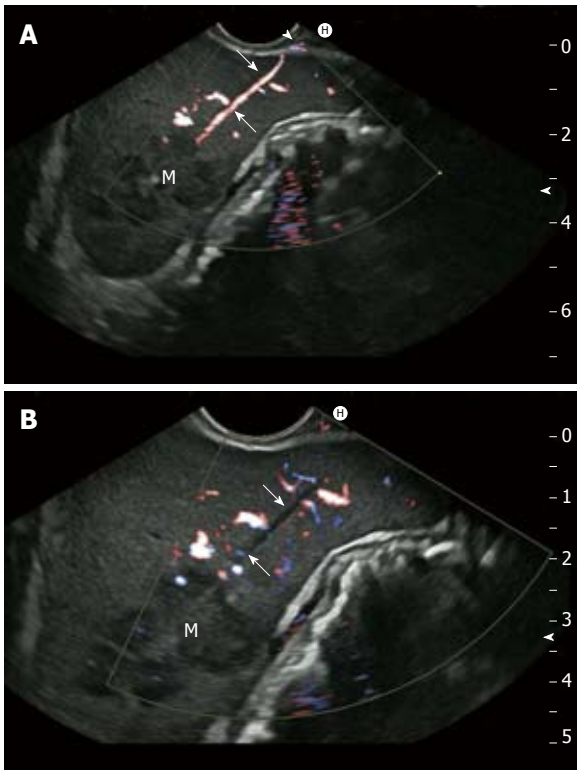


Figure 3 Extraluminal bleeding from needle track following endoscopic ultrasonography-guided fine-needle aspiration. Hemorrhage occurred immediately after endoscopic ultrasonography-guided fine-needle aspiration (22 Gauge) of a splenic metastasis (M) of gastric cancer. A: Note blood flow within needle track (arrows), which extends beyond the splenic capsule (arrowhead); B: Bleeding ceased spontaneously approximately 3 min later (no detectable flow within the needle track; arrows).

following pancreatic mass EUS-FNA. One patient developed uncontrolled bleeding from a pseudoaneurysm and died following EUS-FNB performed using a radial-scanning echoendoscope[129]. Another patient died in the consequence of massive gastrointestinal bleeding 6 h after EUS-FNA of a pancreatic cancer[31]. The third fatal outcome was observed after a 19 G EUS-FNA and intracystic brushing of a cystic pancreatic lesion in a patient receiving anticoagulation therapy. The patient died one month later due to a subacute retroperitoneal bleed related to the EUS-guided procedure and further complications[130].

Abdominal and thoracic pain, pneumothorax and pneumoperitoneum

Several studies have described abdominal pain (following EUS-FNB of pancreatic lesions and abdominal lymph nodes) or thoracic pain (following EUS-FNB of mediastinal lymph nodes and mediastinal mass lesions) without clinical, laboratory, or radiological findings suggestive of any specific complication[30,53,60]. One case of massive pneumoperitoneum due to transduodenal EUS-FNB of a solid pancreatic lesion resolved without any intervention other than antibiotics[131]. In the absence of clinical and radiological evidence of bowel perforation, the authors explained that the pneumoperitoneum resulted from

insufflated air tracking into the peritoneal cavity through the EUS-FNB site. They postulate that some patients with abdominal pain following EUS-FNB have unrecognized pneumoperitoneum[131].

Pneumothorax following EUS-FNB has been reported as one of three complications in a series of 159 EUS-FNBs for transesophageal biopsy of mediastinal masses[132].

Tumor cell seeding

Needle-tract seeding is a rare complication following FNB of intra-abdominal tumors. Based on large retrospective surveys, its frequency is estimated at 0.003% to 0.009%[133]. However, one prospective comparative study reported the incidence of needle-tract implantation of HCC and pancreatic carcinoma after ultrasound-guided percutaneous puncture to be 1.5%[134]. A systematic review and a meta-analysis of percutaneous biopsy of HCC reported tumor seeding frequencies of 2.29%[135] and 2.7%[136], respectively. After percutaneous biopsy of colorectal cancer liver metastases, the risk of needle-track metastases seems even higher, occurring in 10% to 19% of cases[133].

The actual frequency of EUS-FNB-related needle-tract seeding is unknown, but six published case reports have raised concern that the substantial rise of EUS-guided biopsies used to diagnose pancreatic masses and lymphadenopathy may increase the incidence of this complication. In one case, a large gastric wall implantation metastasis with retroperitoneal invasion (50 mm × 30 mm) was diagnosed 16 mo after potentially curative resection of a small (8 mm) T1N0M0 pancreatic tail carcinoma. The tumor had previously been sampled using transgastric EUS-FNA (with five needle passes)[137]. Two similar cases have been reported recently. In both cases, gastric wall metastases occurred at 26 mo and nearly 4 years after transgastric EUS-FNB and pancreatic surgery with curative intent for solid-cystic pancreatic cancer[138,139]. In the fourth case, transgastric EUS-FNB (one needle pass) of a melanoma metastasis to a perigastric lymph node was complicated by a large (30 mm) gastric implantation metastasis along the needle tract. It was detected and resected 6 mo later during surgical resection of lymph node metastasis following neoadjuvant chemotherapy[140]. A recent case report describes needle tract implantation metastasis of the esophageal wall following 19 G EUS-FNA of a large metastatic subcarinal lymph node metastasis of gastric cancer. The esophageal implantation metastasis developed despite perioperative systemic chemotherapy and disappearance of the subcarinal metastatic lymph node; fortunately, it resolved after 2 mo of radiotherapy[141]. The potential risk of peritoneal carcinomatosis due to transgastric EUS-FNA of pancreatic malignancies was highlighted by the case of peritoneal dissemination of an intraductal papillary mucinous neoplasm of the pancreas following EUS-FNA of the tumor[142]. Nevertheless, the risk of peritoneal carcinomatosis appears to be significantly lower with EUS-FNB compared to percutaneous needle biopsy under computed-tomographic or ultrasonographic guidance[143].

Recent data demonstrate vital tumor cell presence within gastrointestinal luminal fluid in 48% of patients with luminal cancers (resulting from tumor cell sloughing), as well as in 10% of patients who underwent EUS-FNB of pancreatic cancer lesions[144]. The importance of this finding with regard to potential tumor cell dissemination by EUS-guided transmural biopsy is speculative at this time. Whereas pancreatic head cancer EUS-FNBs are performed typically through the duodenal wall (which is later resected with the pancreatic head in cases of resectable cancers), pancreatic body and tail tumor EUS-FNBs are performed transgastrically, crossing the peritoneal structures of the Bursa omentalis. Due to the risk of tumor cell seeding along the needle tract, which is proven in principle by the above described case reports, the indication for preoperative EUS-FNB of suspected distal pancreatic cancer is controversial[145-147]. To date, prospective comparative studies of the long-term impact of pre-operative EUS-FNB of suspected pancreatic malignancies are lacking. A recent retrospective study investigated the long-term outcomes of patients undergoing distal pancreatectomy for primary pancreatic neoplasias, comparing patients who received (n = 179) and who did not receive pre-operative EUS-FNB (n = 51)[112]. After a 16-mo median follow-up, no local recurrences were detected within the gastric wall among 91 patients with adenocarcinoma or neuroendocrine tumors who underwent pre-operative EUS-FNB. Median disease-specific survival and recurrence-free survival in patients with pancreatic adenocarcinoma were not statistically worse in 57 patients with pre-operative EUS-FNB as compared with six patients without pre-operative transgastric biopsy. However, partial gastrectomy due to suspected infiltration of the gastric wall by pancreatic adenocarcinoma was performed only in patients with pre-operative EUS-FNB. The study authors expressed concern that surgical pathology showed direct transmural gastric invasion from the pancreatic primary site in six of these seven patients[112].

Postoperative complications

Scarce information exists regarding the potential impact of pre-operative EUS-FNB on complications of subsequent surgery. One study showed no correlation with overall morbidity, specific complications, or technical difficulties during distal pancreatectomy, when analyzed according to specific pancreatic pathology sub-classifications. However, subgroup analysis revealed a higher incidence of postoperative complications in patients with cystic neoplasms who underwent pre-operative EUS-FNB[112].

False-positive results

A false-positive diagnosis obtained from EUS-FNB may result in inappropriate treatment decisions, which could negatively impact the patient's clinical outcome. Therefore, we regard false-positive results as a major EUS-FNB complication in this review. The overwhelming majority of studies report EUS-FNB specificity and positive predictive value for detecting cancer at 100%. A thorough

review of EUS-FNB studies conducted in 2008 identified only ten studies (1750 cases) plus one case study that altogether reported 27 false-positive diagnoses using EUS-FNB[8]. In most of these cases, benign pancreatic diseases (chronic pancreatitis, intrapancreatic splenic heterotopia, mucinous cystic adenoma) were misdiagnosed as ductal adenocarcinoma or neuroendocrine pancreatic tumor[64,66,145,148-154]. However, few studies have correlated EUS-FNB findings with surgical pathology[149,150,155-158]. Therefore, the "true false-positive rate" of EUS-FNB was unknown until a remarkable 2010 study presented a more realistic picture[159]. A cohort of 337 consecutive patients with positive or suspicious EUS-FNB results underwent surgery in the absence of neoadjuvant therapy; there were 19 false-positive cases (5.6%) (based on "positive" cytopathological interpretations) and 25 false-positive cases (7.3%) (based on "positive" plus "suspicious" cytopathological findings), respectively. For pancreatic lesions, the authors reported a 2.2% rate of discordance between preoperative cytological diagnosis and subsequent surgical pathology. In this study, most discordant cases resulted from malignant cell contamination by luminal cancers (primarily esophageal) when performing EUS-FNB of nearby lymph nodes. Retrospectively, only 11 cases were identified as resulting from cytopathological interpretive error[159]. These results are supported by a retrospective cohort study of 367 patients who underwent surgical resection without previous neoadjuvant treatment[160]. This study showed a 1.1% false-positive rate of EUS-FNB for solid pancreatic lesions. When suspicious cytological findings were considered, the false-positive rate was estimated at 3.8%. On review of discordant cases, almost all were caused by pathological misinterpretation in the setting of chronic pancreatitis[160]. Another possible explanation for false-positive diagnosis by EUS-FNB may be inadvertent aspiration of contaminated luminal fluid or traversal of mucosal high-grade dysplasia or malignant infiltration of the gastrointestinal wall[144,161]. Malignant cells are identified within gastrointestinal luminal fluid not only in 48% of cases of luminal cancer, but also in 10% of cases of extraluminal (pancreatic) cancer undergoing EUS-FNB[144].

Safety of pancreaticobiliary tandem procedures and impact of prior biliary stenting on risks associated with EUS-FNB

Due to its high accuracy and lower complication rate compared to ERCP for suspected pancreaticobiliary disease (especially for obstructive jaundice), EUS and EUS-FNB should be performed first when possible. Moreover, biliary stent placement prior to EUS is believed to negatively affect the accuracy of endosonographic staging of pancreatic head malignancies[162]. To perform EUS, EUS-FNB and therapeutic ERCP in one session has advantages with regard to cost, total procedure time and workflow. Several studies have shown that single-session EUS and ERCP does not result in higher complication rates[163-168]. However, ERCP is more widely available than EUS and some cases (e.g., obstructive cholangitis) require biliary decompression more urgently than cytological diagnosis. Therefore, biliary stenting often precedes EUS-FNB. A recent retrospective comparative study showed that pre-EUS stenting of biliary obstructions due to pancreatic cancer did not increase the EUS-FNB complication rate. Moreover, the accuracy of tissue diagnosis was not impaired if ERCP and endoscopic stenting were performed more than one day prior to EUS-FNB[169].

Risk factors for EUS-FNB complications

Due to the relatively low number of complications associated with EUS-FNB, available studies are statistically underpowered to adequately address the question of risk factors. Endosonographer experience seems to be important. Of five nonfatal complications in a large multicenter EUS-FNA study (n = 457), none occurred during the last 12 mo of the study[58]. Similarly, the largest EUS-TCB study (n = 247) showed that all complications were observed among the first 100 patients[56].

There is no evidence that needle size affects complication rate. Only one retrospective multicenter study reported a complication rate of 2% in 540 patients undergoing 22 G EUS-FNA of solid pancreatic masses, versus 0% in 302 patients undergoing 25 G EUS-FNA[113]. Small prospective studies comparing needle sizes reported no complications in any group[170,171]. EUS-FNB using a 19 G needle, when combined with cytobrushing of pancreatic cystic lesions, conveys a significant complication risk associated primarily with cytobrush trauma to the cyst wall. In a prospective controlled study of 37 patients with 39 cystic pancreatic lesions, seven experienced complications (19%): one experienced severe bleeding and one experienced severe pancreatitis[172]. In another study using the same technique (n = 30), three patients experienced complications, including one case of fatal bleeding[130]. This procedure-related morbidity is considerably higher than the 2.2% to 5.2% complication rate reported in several EUS-FNB studies of cystic pancreatic lesions[54,86,87,173]. Nevertheless, the overall EUS-FNB complication rate seems higher for cystic than solid pancreatic masses[54,58,61]. In a systematic review which included 7337 EUS-FNBs of solid pancreatic masses and 909 EUS-FNBs of pancreatic cystic lesions, complications were reported in 60 patients with solid masses (0.82%; only prospective studies: 2.44%) and 25 patients (2.75%; only prospective studies: 5.07%) with pancreatic cystic lesions[61]. Data from the same systematic review confirmed that EUS-FNB is exceptionally safe for mediastinal lesions (n = 1310; complication rate: 0.38%), abdominal masses (n = 381; complication rate: 0.26%) and adrenal gland biopsies (n = 81; complication rate: 0%). EUS-FNB had an associated overall morbidity of 1.03% for pancreatic lesions (n = 8246; only prospective studies: 2.64%), 2.33% for liver lesions (n = 344), 2.07% for perirectal lesions (n = 193), and 3.53% for ascites (n = 85)[61].

Sedation-related complications and safety of propofol-based deep sedation

Safe and effective EUS and EUS-FNB performance re-

quire adequate sedation. Only a few studies report on the frequency of sedation-related complications in EUS and EUS-FNB. In the prospective German EUS registry, oxygen desaturation (SpO2 < 80%) was reported in 0.39% of patients, but most patients (94.4%) did not require intubation or mechanical ventilation[32]. A retrospective single-center study on the safety of nurse-administered propofol sedation for upper gastrointestinal EUS showed that 6 of 806 patients (0.7%) experienced declines in oxygen saturation (SpO2 < 90%), and four patients (0.5%) required assisted positive pressure ventilation[17]. Comparable results have been reported by other authors[174]. Several studies show that EUS with deep sedation using propofol (administered by an anesthesiologist, by a second gastroenterologist, or by a trained nurse) is as safe as conscious sedation using benzodiazepines (midazolam) and pethidine[174-177].

PREVENTION OF EUS-RELATED COMPLICATIONS

Strategies aimed to minimize potential risks of EUS and EUS-FNB include: (1) adequate education and examiner training; (2) familiarity of the endosonographer with the results of previous investigations and of possible therapeutic implications of EUS and EUS-FNB results; (3) appropriate consideration of the patient's clinical condition and of indications and contraindications for EUS and EUS-FNB; (4) careful consideration of a meaningful clinical strategy for investigating and treating each patient; and (5) optimization of prerequisites and conditions for the planned investigation or procedure[40].

We suggest the following measures, which seem appropriate to minimize the risks of EUS and EUS-FNB: (1) participation in supervised training programs in EUS and EUS-guided interventions. EUS and EUS-FNB are complex, technically demanding procedures in which the findings are difficult to interpret and which require advanced endoscopic skills, outstanding experience in diagnostic ultrasonography, and a fundamental understanding of the anatomy of the gastrointestinal tract and surrounding structures. The guidelines for credentialing and granting privileges for endoscopic ultrasound published by the American Society for Gastrointestinal Endoscopy recommend a minimum of 150 supervised EUS investigations, including 75 pancreatobiliary EUS investigations and 50 EUS-FNA procedures, to establish comprehensive competence in all aspects of EUS[178]; (2) specific guidelines for EUS training should be implemented in national gastroenterological societies' training programs[7,179,180]. Curricula of formal training programs should include sessions on complications and contraindications of EUS and EUS-FNB; (3) EUS and EUS-FNB should only be carried out in cooperative and adequately sedated patients. Oxygen saturation, heart rate, blood pressure and (whenever possible and necessary) electrocardiography should be monitored[181-183]; (4) water filling in the upper gastrointestinal tract should be restricted to the minimum amount necessary and the endoscopy unit and team should be prepared to perform nasopharyngeal suction; (5) in EUS examinations of the pancreas, biliary duct, ampulla, and duodenum in patients with biliary stents, the correct position of the stent should be checked prior to examination; (6) in the case of stenosing tumors and for specific anatomical locations (e.g., the tip of the duodenal bulb), care should be taken when maneuvering the echoendoscope. Dilation to achieve echoendoscope passage through malignant strictures should be carried out only in selected cases; (7) EUS and EUS-FNB are contraindicated in all patients and in all conditions in which the risks of the procedure outweigh the expected benefits of its diagnostic information (Table 5)[8,180]. According to recent guidelines, EUS-FNB of solid masses and lymph nodes may be performed in patients taking ASA or NSAIDs, but not in patients receiving: thienopyridines (clopidogrel, prasugrel, ticagrelor), oral anticoagulation, standard heparin treatment, LMWH or fondaparinux in therapeutic doses. EUS-FNB of cystic lesions should be avoided in patients taking any antiplatelet agent (e.g., ASA, thienopyridines). If EUS-guided sampling is indicated in a patient on LWMH, the biopsy should be performed no earlier than 8 h following the last administration of the drug[180,189,190]; (8) whenever possible, color-coded duplexsonography (CCDS) should be used before puncture to avoid vessels and highly vascularized structures in the needle trajectory; (9) before attempting EUS-FNB of large, solid-appearing mediastinal mass lesions, all appropriate methods (e.g., CCDS, contrast-enhanced endosonography (CE-EUS) and real-time elastography) should be used to exclude cystic lesions; (10) transgastric EUS-FNB of pancreatic masses should be restricted to those patients in whom definitive cytopathological diagnosis is likely to alter man-

Table 5 Contraindications to endoscopic ultrasonography and endoscopic ultrasonography-guided fine needle biopsy

Contraindications to EUS and EUS-FNB
Lack of informed consent[1]
Lack of cooperation or insufficient sedation[1]
Results of examination are unlikely to have a significant clinical impact[1]
Anticoagulation treatment or non-substituted coagulopathy (international normalized ratio > 1.5; platelet count < 50 000/mL; heparin administration at therapeutic doses)[2]
Inhibition of platelet aggregation by clopidogrel and other thienopyridines[2]
Failure of ultrasonographic needle control[2]
EUS-FNB of lesions upstream of a bile duct stricture which is insufficiently drained[2]
Cystic mediastinal and pelvic lesions[2,3]
Interposition of vessels[2,4]

[1]Contraindication to endoscopic ultrasonography (EUS) and endoscopic ultrasonography-guided fine needle biopsy (EUS-FNB); [2]contraindication to EUS-FNB; [3]relative contraindication, peri-interventional antibiotic treatment is mandatory; [4]relative contraindication, benefit has to be weighed carefully against risk. Transaortic endoscopic ultrasonography-guided fine needle aspiration of mediastinal lymph nodes and tumors[184,185] of the pericardium and a left atrial mass[186], and of intravascular tumors[187,188] have been reported without any major complications.

Table 6 Morbidity of diagnostic endoscopic ultrasonography compared to other endoscopic procedures (%)

Complications	Total	Bleeding	Perforation
Diagnostic esophagogastroduodenoscopy	0.009-0.2	0.002-0.06	0.0009-0.04
Diagnostic colonoscopy	0.02-0.25	0-0.03	0.005-0.2
Colonoscopy + polypectomy	0.36-9.7	0.26-8.6	0.06-1.1
Diagnostic ERCP	1.38	0	0.11
Therapeutic ERCP	5.0-9.8	0.49-2.0	0.3-0.8
EUS	0.03-0.15	0-0.03	0.03-0.15
EUS-FNB	0.3-6.3	0.15-3.7	0-0.86

Complication risk of non-interventional diagnostic endoscopic ultrasound (EUS) and endoscopic ultrasonography-guided fine-needle biopsy (EUS-FNB): compared to other endoscopic examinations and interventions (pooled data from literature, table adapted from: Jenssen, Mayr, Faiss and Nuernberg[40]). ERCP: Endoscopic ultrasonography-guided trucut biopsy.

agement significantly. In general, there is no indication for EUS-FNB of a pancreatic mass lesion that is typical of adenocarcinoma in patients who have no criteria of non-resectability[8,9,147,180]. Advanced EUS techniques (e.g., CCDS, CE-EUS, real-time elastography) may help to select resectable pancreatic masses that are atypical of ductal pancreatic adenocarcinoma[191-197]; (11) trucut needles should be used with caution in selected cases and indications. Although a prospective randomized study found no significant difference in the complication rates between EUS-TCB and EUS-FNA[57], the use of EUS-TCB is limited by technical drawbacks, especially for pancreatic head lesions[198]. Subepithelial tumors, unexplained gastrointestinal wall thickening, suspected malignant lymphoma, and suspected autoimmune pancreatitis are now emerging as special indications for EUS-TCB[8] or, alternatively, for a new type of aspiration needle that is suited to obtain core particles for histological evaluation[199]; (12) antibiotic prophylaxis should be administered in patients undergoing EUS-FNB of cystic lesions and fluid collections[88,100,180]. Some experts also recommend peri-procedural antibiotic prophylaxis for EUS-FNB of the perirectal space[200] and for EUS-TCB of subepithelial tumors[76]. However, there are no valid data supporting routine use of antibiotics for these indications; and (13) complications should be assessed in a prospective manner, and all complications should be analyzed. Prospective risk assessment is prerequisite to the prevention and analysis of complications. Careful prospective studies show higher EUS-FNB complication rates compared to retrospective studies (2.44% *vs* 0.35%)[61] (Table 4). The American Society for Gastrointestinal Endoscopy recommends that specific quality indicators (e.g., indications, sedation, immediate and delayed complications, procedure success) should be tracked routinely in all patients undergoing EUS[201].

In conclusion, diagnostic EUD is a safe procedure. The EUS complication rate of 0.03% to 0.15% is comparable to that of upper gastrointestinal tract diagnostic endoscopy (Table 6)[40,88,180]. Due to specific mechanical and optical properties of echoendoscopes, the risk of esophageal or duodenal perforation seems somewhat higher compared to esophagogastroduodenoscopy. The major risks of EUS-FNB are pain, acute pancreatitis, infection and hemorrhage[61]. Tumor cell seeding is exceedingly rare. The complication rate of EUS-FNB is 0.3% to 6.3%, which is comparable to that of colonoscopy with polypectomy (Table 6). Very few fatal complications of diagnostic EUS have been reported (overall mortality 0.02%)[31,38,61]. Adherence to guidelines on indications and contraindications, adequate education and training, prospective registration, and careful assessment of all complications are essential preconditions for enhancing safety and efficacy of EUS and EUS-FNB. A constructive process of continuous quality and safety assessment should be implemented in all centers performing endoscopic endosonography.

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REVIEW

Hepatitis B in pregnancy

Guglielmo Borgia, Maria Aurora Carleo, Giovanni Battista Gaeta, Ivan Gentile

Guglielmo Borgia, Maria Aurora Carleo, Ivan Gentile, Department of Public Medicine and Social Security, Section of Infectious Diseases, University of Naples "Federico II", 80131 Naples, Italy
Giovanni Battista Gaeta, Department of Internal Medicine, Section of Infectious Diseases, Second University of Naples, 80131 Naples, Italy
Author contributions: Gentile I and Carleo MA drafted the manuscript; Gaeta GB drafted part of the manuscript and revised the entire manuscript; Borgia G drafted part of the manuscript, revised it and was coordinator of the writing team.
Correspondence to: Guglielmo Borgia, MD, Professor of Infectious Disease, Department of Public Medicine and Social Security, Section of Infectious Diseases, Ed. 18, University of Naples "Federico II", Via Sergio Pansini 5, 80131 Naples, Italy. borgia@unina.it
Telephone: +39-81-7463083 Fax: +39-81-7463190
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Abstract

Chronic hepatitis B virus (HBV) infection affects about 350 million individuals worldwide. Management of HBV infection in pregnancy is difficult because of several peculiar and somewhat controversial aspects. The aim of the present review is to provide a tool that may help physicians to correctly manage HBV infection in pregnancy. This review focuses on (1) the effect of pregnancy on HBV infection and of HBV infection on pregnancy; (2) the potential viral transmission from mother to newborn despite at-birth prophylaxis with immunoglobulin and vaccine; (3) possible prevention of mother-to-child transmission through antiviral drugs, the type of antiviral drug to use considering their efficacy and potential teratogenic effect, and the timing of their administration and discontinuation; and (4) the evidence for the use of elective caesarean section *vs* vaginal delivery and the possibility of breastfeeding.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection affects about 350 million individuals worldwide: half of them acquired the infection either perinatally or in early childhood, especially in endemic areas[1,2]. The estimated burden of death associated with HBV infection is 500 000-1.2 million per year. Progression of the disease towards cirrhosis, liver failure or hepatocellular carcinoma occurs in 15%-40% of infected subjects. The risk of chronic infection is inversely proportional to the age at which the infection was acquired[3].

HBV infection in pregnancy has several peculiar aspects among which are the effect of pregnancy on HBV infection, the potential viral transmission from mother to newborn and its possible prevention through antiviral drugs, and the potential teratogenic effect of these drugs. These elements can complicate the management of HBV infection in the setting of pregnancy[4]. The aim of this review was to examine recent studies devoted to the clini-

cal, therapeutic, and prognostic aspects of HBV infection during pregnancy in order to extrapolate and interpret data that can help physicians in the management of HBV infection in this setting.

EFFECT OF HBV INFECTION ON PREGNANCY

In most cases, acute or chronic HBV infection in pregnancy is similar to that in the general adult population: HBV infection does not increase the mortality and it does not yield teratogenic effects. However, a higher incidence of low birth weight and prematurity has been reported during acute infection than in the general population[1], whereas gestational diabetes mellitus, antepartum hemorrhage and preterm delivery are more frequent in chronic maternal HBV infection than in the general population[1]. Acute infection must be differentiated from other acute liver diseases that occur during pregnancy such as intrahepatic cholestasis[1].

Differently, the management of more advanced disease (cirrhosis) may be troublesome. Patients with advanced cirrhosis usually are amenorrheic and infertile due to hypothalamic-pituitary dysfunction, but successful pregnancy may be completed in those with well-compensated disease[5]. In these cases, increased maternal and fetal problems can be expected in about 50% cases, with increased fetal loss[5]. The main risk to the mother is rupture of esophageal varices and consequent bleeding (20%-25%) especially during the second trimester or during labor. Other risks include hepatic decompensation, jaundice, and rupture of splenic aneurysms[5]. In the presence of known esophageal varices, women planning a pregnancy should be considered for endoscopic therapy, shunt surgery, or even liver transplantation before pregnancy. Even if varices are absent before pregnancy, all patients should undergo upper endoscopy for assessment of varices in the second trimester and, if large varices are present, beta blocker therapy must be administered despite occasional fetal effects[5]. Acute variceal bleeding is managed endoscopically, as in the non-pregnant setting, though vasopressin is contraindicated. Ascites and hepatic encephalopathy are treated in the usual way (i.e., use of diuretics, rifaximin, lactulose, *etc.*). Except in patients with known large varices, a vaginal delivery is preferable to avoid abdominal surgery[5].

Pregnancy in a liver transplant recipient represents a unique clinical situation: with the success of liver transplantation, more pregnancies are being reported in liver recipients, and a carefully planned pregnancy in a stable healthy patient, beyond the first 2 years after orthotopic liver transplantation, can have an excellent outcome for fetus, mother, and the graft. However, this is still a high-risk pregnancy with increased fetal prematurity and dysmaturity[5]. In addition, it entails some risk to the allograft from acute cellular rejection or recurrent viral hepatitis[5,6].

EFFECT OF PREGNANCY ON HBV INFECTION

During pregnancy there are several modifications in the maternal immune system, namely, a shift in the Th1-Th2 balance towards a Th2 response, increased amounts of regulatory T cells, *etc.*, that contribute to a depressed immune response against HBV. The aim of these modifications is to prevent the rejection of the fetus who is partially allogenic for the mother's immune system. These modifications result in an increase of HBV DNA and a reduction of aminotransferase levels. After delivery the immune system is restored thereby causing opposite consequences; namely, there is a significant increase in alanine aminotransferase (ALT) and a reduction of HBV DNA in this period[7].

ter Borg *et al*[7] studied the course of the liver disease in 38 HBV-positive pregnancies before, during and after delivery. In 13 pregnancies, antiviral therapy with lamivudine was started during the last trimester of pregnancy due to high viremia to reduce the risk of mother-to-child transmission (see later) and was stopped immediately after delivery. Forty-five per cent of untreated women experienced a flare (defined as a three-fold increase in ALT) within 6 mo after delivery, compared to 62% of women who received lamivudine. No clinical decompensation occurred in these women. However, the authors recommend monitoring closely and, if necessary, administering treatment to women with chronic HBV shortly after delivery.

PERINATAL HBV TRANSMISSION

Perinatal transmission is a common mode of HBV transmission worldwide[1]. HBV infection in newborns is defined as hepatitis B surface antigen (HBsAg) positivity 6 mo after birth. Antibody to hepatitis B e antigen (anti-HBe) and anti-hepatitis B core antigen cross the placental barrier and disappear in nearly all babies before 12 and 24 mo of age, respectively[8]. Therefore, they simply represent the transplacental maternal antibodies and are not indicators of HBV infection status[8].

Without prophylaxis the risk of mother-to-child transmission is very high. It varies with the HBeAg/anti-HBe status of mothers, being 70%-90% for HBeAg-positive mothers, 25% for HBeAg-negative/HBeAb-negative mothers and 12% for HBeAg-negative/anti-HBe-positive mothers[9-12]. Maternal screening programs aimed at identifying HBsAg-positive mothers are part of pregnancy routine examinations in most countries. Once HBsAg-positive mothers are identified, their babies receive passive-active immunoprophylaxis at birth to reduce vertical HBV transmission[3]. Passive immunoprophylaxis consists of the administration of hepatitis B immune globulin (HBIG) whereas active immunoprophylaxis is the administration of hepatitis B vaccine[1,3].

Although this prophylaxis is effective in blocking HBV maternal transmission, a small but not negligible proportion of children (3%-13%) born from HBsAg-positive mothers, especially those carrying HBeAg, become HBsAg carriers despite correct passive-active immunoprophylaxis[1,10,12-14].

The biological explanation for the higher risk of transmission for HBeAg-positive mothers is that, differently from HBsAg, maternal HBeAg could pass through placenta from mother to fetus and induce T-cell tolerance *in utero*[8]. The intra-uterine infection of HBV, whose mechanism remains unclear, is the major cause of unsuccessful immunological blockade. High levels of serum HBV DNA in pregnant women is the main risk factor for the occurrence of HBV intra-uterine infection: it correlates with the cord blood HBV DNA level and HBsAg titer[12,15]. It has also been demonstrated that HBV can infect all kinds of cells in the placenta (decidual, trophoblastic, villous mesenchymal, villous capillary endothelial cells) and that HBV DNA is present in all generations of spermatogenic cells and sperms in HBV-infected males, in follicular fluid and in ovary (cellular transfer)[15,16]. The presence of the virus in the spermatogenic cells can have a role in the transmission of HBV infection to the newborn, as a high homology between the father's and the child's viral sequences in discordant couples has been found[16].

The maternal HBeAg-positive serological status and high viral load are associated positively with intrauterine transmission of HBV, especially through villous capillary endothelial cells. In fact, HBeAg can pass through the placenta *via* partial placental leakage or *via* the "cellular route"[17]. The absence of HBeAg expression is associated with lower levels of viral replication and with a significantly lower risk of intrauterine transmission of HBV[17].

TREATMENT OF HBV DURING PREGNANCY

All decisions about the treatment of HBV in pregnancy must include an analysis of the risks and benefits for the mother and fetus. The major issue regarding the mother is the consequences of the treatment on short- and long-term liver disease outcomes. The major concern for the fetus is the risk of exposure to potentially teratogenic drugs during early embryogenesis[18].

Seven drugs have been approved by the United States Food and Drug Administration (FDA) for the treatment of hepatitis B: PEG-interferon alpha 2a, interferon alpha 2b, lamivudine, adefovir, entecavir, telbivudine and tenofovir[3] (Table 1).

Interferon, contraindicated during pregnancy, can be used in women of childbearing age because it is usually given for a defined period (48-96 wk). The administration of interferon must be accompanied by the recommendation to use contraception during treatment[3,18].

The oral antiviral agents, namely nucleoside or nucleotide analogues that inhibit viral polymerase, are generally used for long periods. However, they also interfere with replication of mitochondrial DNA thereby resulting in potential mitochondrial toxicity; effects of which are poorly known in the developing fetus[18].

Table 1 Pregnancy category of antiviral drugs against hepatitis B virus

Drug	Pregnancy category
Lamivudine	C
Entecavir	C
Telbivudine	B
Adefovir	C
Tenofovir	B
Interferon alpha 2b	C
Pegylated-Interferon alpha 2a	C

The FDA classifies drugs in five categories (A, B, C, D and X) according to their possible teratogenic effects in humans or animal models. The 5 oral nucleos(t)ide analogues for HBV treatment are classified as either a category B or a category C agent. Category C drugs, namely lamivudine, adefovir and entecavir, are those that exert teratogenic or embryocidal effects in animals and for which there are no controlled studies in humans[16]. Lamivudine is highly toxic in rabbits with first trimester exposure; however, because it was the first oral agent approved for the treatment of HBV, extensive clinical experience indicates a general lack of teratogenetic effects in humans[18].

Category B drugs, namely telbivudine and tenofovir, are those that, according to the results of animal studies, carry no teratogenic or embryogenic risk and for which there have been no controlled human studies or for which animal studies may indicate a risk but controlled human studies refute these findings[19]. Tenofovir has both a high power and a high genetic barrier to resistance. Telbivudine has a high power, but a low barrier to resistance[18].

Safety data on HBV antivirals during pregnancy come from two major sources: the Antiretroviral Pregnancy Registry (APR) and the Development of Antiretroviral Therapy Study (DART)[20,21], due to the fact that some analogues are active both against HBV and against human immunodeficiency virus (HIV). The APR, an international, voluntary, prospective registry, has analyzed as of January 2010 a cohort of 11 867 women exposed to antiretroviral therapies, most of whom are HIV-1 monoinfected and only 112 of whom are HBV monoinfected. The results indicate that the rate of birth defects among women exposed to HBV therapy (2.7% of live births) is similar to that in the general population (2.72% rate) as reported by the Centers for Disease Control and Prevention (CDC) birth defect surveillance system. No significant difference was reported in the rate of adverse outcomes if the initial exposure of any HBV drug occurred in the first trimester (2.7%) compared to the second or third trimester (2.5%) of pregnancy. Lamivudine and tenofovir are the two agents with the most *in vivo* experience in the first trimester, and these appear to be

safe. For telbivudine and entecavir, only 5 and 12 pregnancies with exposure in the first trimester are recorded in this registry, with no adverse outcomes reported. The APR has some limitations: short follow-up and recording only defects identified at birth. Therefore, developmental anomalies (e.g., cardiac or neurologic defects) identified at a later date may be omitted[19].

The DART study is a 6-year, multicenter, randomized trial of antiretroviral therapy among adults with symptomatic HIV-1 infection or advanced disease/AIDS in Africa. The rate of congenital anomalies reported in this study is 3% and compares favorably to the 2.72% reported by the CDC birth defect surveillance system[21].

It is difficult to decide what anti-HBV therapy to administer in pregnant women. In addition to parameters usually considered in decision-making about hepatitis B therapy (age, stage of disease, comorbidity, viral load, genotype, power of the agent, genetic barrier to resistance, *etc.*), specific issues in the treatment choice for women of childbearing age include safety of drugs in pregnancy and breastfeeding, and proposed length of therapy[1].

In the case of untreated women who are "planning" a pregnancy, it may be prudent to delay therapy until after the child is born. For example, if the woman is in the immune-tolerant phase of infection (high HBV DNA levels with normal ALT and inactive liver biopsy), treatment can usually be postponed until after delivery. However, should the woman be HBeAg-positive with a high viral load, a 3rd trimester prophylaxis to reduce transmission could be initiated in case of pregnancy (see later)[3].

For pregnant women already in treatment, in the case of significant fibrosis, therapy should be continued to reduce the risk of decompensation of liver disease. This would have a negative impact on the health of the fetus. In this case, it is preferable to switch to an agent that is safe in pregnancy and which has a long *in vivo* experience in this setting[5].

In summary, the choice of the anti-HBV drug to administer in a pregnant woman depends on whether the aim of treatment is to treat an active liver disease for which therapy cannot be delayed, or to prevent transmission of infection to the fetus from high viremia mothers without significant liver disease. A third decision-requiring condition is that of women who become pregnant while on anti-HBV therapy in whom the drug can be continued, stopped or changed into a class B drug.

PREVENTION OF MOTHER-TO-CHILD HBV TRANSMISSION WITH ANTIVIRAL DRUGS

There is no consensus about the therapy choice for pregnant women who are HBsAg-positive and highly viremic in the third trimester to prevent perinatal transmission[18].

In a pilot study published in 2003, eight women with HBV DNA levels greater than 10^9 copies/mL were given 150 mg of lamivudine daily during the last month of gestation: babies received passive and active immunization and only one became infected, compared to 7 of 25 (28%) cases of transmission in a matched historical control population[22].

A randomized, double-blind, placebo-controlled trial conducted with 150 HBsAg-positive highly viremic pregnant women evaluated whether lamivudine administered from the 32nd week of gestation to 4th week post-partum prevented HBV transmission to newborns who received standard prophylaxis with HBIG and vaccination. All but one woman were HBeAg-positive. At 1 year of age, 18% of babies from lamivudine-treated mothers were HBsAg-positive compared to 39% in the placebo-treated arm in intention-to-treat analysis ($P = 0.014$). Based on these results, Xu *et al*[23] recommended treatment in the third trimester for women with high viral loads. However, these data should be interpreted with caution due to a high dropout rate (13% in the lamivudine group and 31% in the placebo group). No safety concerns were noted in the lamivudine-treated mothers and their infants.

A recent meta-analysis of 10 randomized controlled trials for a total of 951 HBV-positive mothers evaluated the efficacy of lamivudine in reducing *in utero* transmission of HBV[24]. The results confirm the safety of lamivudine and its efficacy in preventing HBV intrauterine infection and mother-to-child transmission. However, the limitation of meta-analysis is the different quality of the studies included and the heterogeneity of the cut-off values for high viral load that prompted therapy[24].

A non-randomized, controlled trial that included 190 HBsAg-positive, HBeAg-positive pregnant women with high viremia (> 10^6 copies/mL) evaluated the efficacy and safety of telbivudine *vs* placebo. In both groups, newborns were treated with standard active/passive prophylaxis within 24 h from birth. Telbivudine was given to mothers from 20 to 32 wk before delivery and was continued for 4 wk after delivery in the case of inactive disease and for 28 wk in the case of active chronic hepatitis. At birth, a significant reduction of HBsAg positivity rate was observed in babies born from telbivudine-treated mothers compared to the control group (6.3% *vs* 30.4%). Twenty-eight weeks after birth, the rate of HBsAg or HBV DNA positivity was significantly reduced in babies born from telbivudine-treated mothers compared to placebo-treated ones (2% *vs* 13%). Post-partum ALT flares occurred in 7.5% of telbivudine-treated women and in 18.5% of placebo-treated women. No safety concerns were observed for mothers or children in either group. No cases of severe hepatitis were observed in women who discontinued telbivudine at week 4 post-partum. These data confirm the need of antiviral therapy in HBeAg-positive pregnant women with high viremia (> 10^6 copies/mL) and the efficacy and safety of telbivudine in this setting[14].

Few data are available about the efficacy of tenofovir in pregnant women. Some information regarding toxicity of tenofovir comes from studies on animals, such

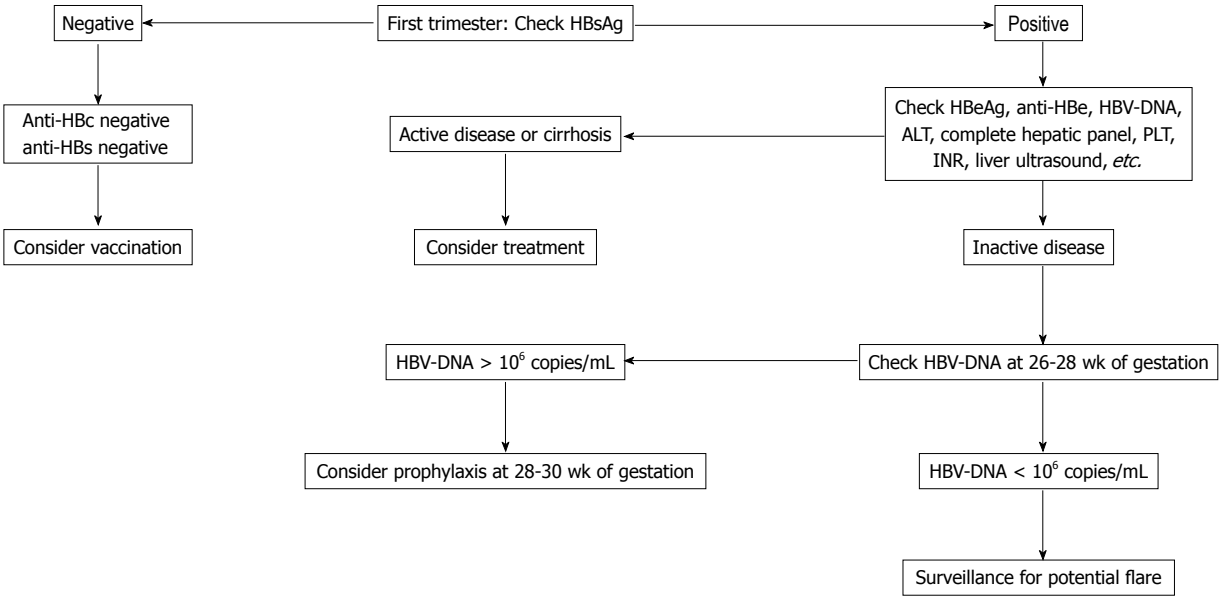


Figure 1 Algorithm for management of hepatitis B virus infection during pregnancy. HBV: Hepatitis B virus; ALT: Alanine aminotransferase; PLT: Platelet count; INR: International normalized ratio; Anti-HBe: Antibody to hepatitis B e antigen; Anti-HBc: Anti-hepatitis B core antigen; anti-HBs: Antibodies to hepatitis B surface antigen.

as gravid rhesus monkeys, which found a reduction of circulating insulin-like growth factor in newborns and severe growth reduction in approximately 25% of the infant monkeys within 2 mo of maternal treatment[25-27]. However, given its potency and its high barrier to resistance, tenofovir would be expected to be at least equally as effective as lamivudine in reducing perinatal transmission; it is an attractive agent to consider in the third trimester[18].

Regarding the issue of an HBV-infected male spouse who is planning a baby, no data are available in terms of drug safety to the fetus.

MANAGEMENT STRATEGY

A proposed strategy in summarized in Figure 1. Briefly, every woman in the first trimester should be screened for HBV infection. If she is negative, she does not have to be routinely vaccinated during pregnancy, although it is considered safe and should therefore be administered to those with high risk behavior for acquisition. Her child will be vaccinated for hepatitis B together with other routine vaccines. If the pregnant woman is positive in early pregnancy, it is necessary to understand the status of the disease. If she has very active HBV (significantly elevated ALT with a high viral load), or if cirrhosis is suspected, therapy should be initiated regardless of trimester. If the woman has inactive disease (low ALT and low viral load), therapy is not warranted and continued surveillance is suggested because of the risk of a flare of hepatitis B later in pregnancy and for several months postpartum[3,18].

Quantification of HBV DNA is recommended in all infected women at the end of the second trimester (at 26-28 wk of gestation): if the viral load is $>10^6$ copies/mL, antiviral prophylaxis of HBV transmission to newborn can be initiated early in the third trimester (28-30 wk), even though there is no consensus or guidelines on this topic, with consideration to the potential mitochondrial toxicity of all nucleoside/nucleotide analogues. Similarly, there is no consensus regarding discontinuation of the drug and the time of discontinuation. The decision should take into account the pre-pregnancy stage of liver disease because of the potential risk of flare. In the absence of active disease or cirrhosis at baseline, treatment discontinuation 4 wk after delivery is reasonable. If the patient has advanced liver disease we do not recommend discontinuation. It should be stressed that breastfeeding while on treatment for HBV is not recommended (see later)[3,18].

ELECTIVE CAESAREAN SECTION *VS* VAGINAL DELIVERY

A meta-analysis of four randomized trials, involving 789 pregnant women, assessed the efficacy and safety of elective caesarean section (ECS, caesarean section before labor or before ruptured membranes) *vs* vaginal delivery in preventing mother-to-child HBV transmission. It was found that ECS *vs* vaginal delivery significantly reduced the rate of maternal transmission of HBV (ECS: 10.5%; vaginal delivery: 28.0%) (relative risk: 0.41, 95% CI 0.28 to 0.60, $P < 0.000\,001$)[28]. No data regarding maternal morbidity or infant morbidity according to mode of delivery were available. However, the conclusions of the meta-analysis must be viewed with great caution given the high risk of bias in each study included in the analysis. The role of ECS in preventing mother-to-child transmission of HBV is uncertain. Currently, there is no convincing evidence that ECS reduces the rate of mother-to-child transmission of HBV compared with vaginal delivery[28].

BREASTFEEDING BY HBV-INFECTED WOMEN

In 1975, before the availability of neonatal immunization, Beasley *et al*[29] reported a rate of HBV acquisition of 53% in breast-fed and of 60% in formula-fed babies born to HBsAg-positive mothers. With the introduction of immunoprophylaxis, a similar rate of infection in breast-fed and formula-fed infants (0% and 3%) was found[30]. Although the high vertical transmission rates confounded the true rate of acquisition from breastfeeding, the current guidelines state that breastfeeding is not contraindicated in HBV-infected mothers who are not on antiviral therapy and whose infants receive immunoprophylaxis[31]. For mothers on antiviral therapy with lamivudine or tenofovir, breastfeeding is not recommended because few data are available about the safety of antiviral exposure during breastfeeding[18].

CONCLUSION

Testing for HBsAg is recommended for every pregnant woman, regardless of previous testing or vaccination. Identification of HBV-positive pregnant women remains the most effective way to prevent HBV transmission to newborns thanks to a very effective passive/active prophylaxis at birth. However, in the case of women with very high viremia, a non-negligible proportion of newborns can acquire the infection (probably through *in utero* transmission) despite the use of passive/active prophylaxis. For this reason, antiviral treatment in the third trimester can be considered for those women. The choice of antiviral should be restricted to those drugs considered safe in this setting. Decisions regarding the time of eventual discontinuation should consider the stage and activity of liver disease and of infection, taking into account also the risk of post-partum hepatitis flare. Breastfeeding is not contraindicated for HBV patients. However, it is not recommended for women taking antiviral drugs. Finally, there is no clear evidence that ECS reduces the risk of mother-to-child transmission compared to vaginal delivery.

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ORIGINAL ARTICLE

Gastro protective properties of the novel prostone SPI-8811 against acid-injured porcine mucosa

Meghali Nighot, Adam Moeser, Ryuji Ueno, Anthony Blikslager

Meghali Nighot, Anthony Blikslager, Departments of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607, United States
Adam Moeser, Departments of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607, United States
Ryuji Ueno, Departments of Sucampo Pharmaceuticals, Inc., Bethesda, MD 20810, United States
Author contributions: All the authors contributed equally to this manuscript.
Supported by Sucampo Pharmaceuticals Inc., Bethesda, MD
Correspondence to: Dr. Anthony Blikslager, Departments of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607, United States. anthony_blikslager@ncsu.edu
Telephone: +1-919-5137725 Fax: +1-919-5136336
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Abstract

AIM: To evaluate the protective properties of novel prostone ClC-2 agonist SPI-8811 in porcine model of gastric acid injury.

METHODS: Porcine gastric mucosa was mounted in Ussing chambers and injured by bathing mucosal tissues in an HCl Ringer's solution (pH = 1.5) with or without SP1-8811 (1 μmol/L), cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor (inhibitor 172, 10 μmol/L, apical) and ClC-2 inhibitor $ZnCl_2$, 300 μmol/L, apical), on the apical surface of tissues. Transepithelial resistance and mucosal-to-serosal ^{3}H-mannitol fluxes were measured over a 90-min period. Tissues were analyzed by morph metric techniques, Immunofluorescence and by western blots.

RESULTS: Compared with control tissues, acid exposure decreased transepithelial electrical resistance (TER) and increased ^{3}H-mannitol flux. Pretreatment of gastric mucosa with SPI-8811 was protective against acid-induced decreases in TER (TER, 50 $\Omega \cdot cm^2$ *vs* 100 $\Omega \cdot cm^2$) and abolished increases in flux (^{3}H-mannitol flux, 0.10 μmol/L·cm^2 *vs* 0.04 μmol/L·cm^2). Evidence of histological damage in the presence of acid was markedly attenuated by SPI-0811. Immunofluorescence and western analysis for occludin revealed enhanced localization to the region of the tight junction (TJ) after treatment with SPI-8811. Pretreatment with the ClC-2 inhibitor $ZnCl_2$, but not the selective CFTR inhibitor 172, attenuated SPI-8811-mediated mucosal protection, suggesting a role for ClC-2. Prostone may serve both protective and reparative roles in injured tissues.

CONCLUSION: ClC-2 agonist SPI-8811 stimulated enhancement of mucosal barrier function by protecting TJ protein occludin in porcine gastric mucosa and thus protected the gastric acid injury in porcine stomach.

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Key words: Stomach; Mucosal permeability; ClC-2 chloride channel; Tight junction

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INTRODUCTION

It is becoming increasingly evident that many patients suffer from gastric ulcers, particularly in groups of pa-

tients such as those in intensive care facilities[1-4]. This suggests that medications that provide gastro protection have the potential to reduce morbidity associated with gastric ulceration. For decades, agents that suppress acid secretion[5-7] have been widely used for treatment of gastric ulcers[1,7-10]. Gastric ulcer disease and repair is complex, involving inflammation, cell proliferation, formation of granulation tissue, and angiogenesis[3,4,11]. However, gastro protection has also been studied in depth. For example, studies with rebamipide or misoprostol, geranylgeranyl hydrochloride (HSP70) have revealed that these compounds have gastro protective properties as well enhancing ulcer healing[2,7,12]. Nonetheless, our understanding of the mechanisms of gastro protection is incomplete and there is the prospect of novel pharmacological agents, aside from antacids, proton pump inhibitors, and prostanoid activators or analogs, which might protect the stomach[12,13].

The gastric barrier is composed of a single layer of columnar epithelium, mucus, bicarbonate layer, and intraepithelial tight junctions (TJs) residing at the apicalmost region of the paracellular space. The gastric barrier serves as the first line of defense against a hostile luminal environment. The mucus bicarbonate barrier is the only pre epithelial barrier between lumen and the epithelium[14,15]. When it is overwhelmed or breaks down in disease, the next series of protective mechanisms come into play, including intracellular neutralization of acid, rapid epithelial repair, and maintenance and distribution of mucosal blood flow. There are two components of this innate mucosal defense: mechanisms that reduce the ability of pathogens and their toxins to invade the mucosa, and the mechanisms that ensure rapid repair of defects in the epithelial monolayer[3,4,15-17]. The next line of mucosal defense is formed by a continuous layer of surface epithelial cells which secrete mucus and bicarbonate and generate prostaglandins, heat shock proteins, refoil factor peptides, and cathelicidins. Because of the presence of phospholipids on their surfaces, these cells are hydrophobic, repelling acid- and water-soluble damaging agents. Interconnected by TJs, the surface epithelial cells form a "barrier" preventing back diffusion of acid and pepsin. Once the epithelial barrier is disrupted, epithelial repair mechanisms must rapidly re-form a continuous epithelial monolayer in order to prevent entry of protons and bacteria[3,4,14,15,18]. The remarkable phenomenon of epithelial restitution during which epithelium re-seals mucosal defects in the presence of acidic environment has been a subject of several studies.

Acid injury is an important component of gastric ulcer disease, despite more recent findings on the role of *Helicobacter pylori* which also contributes to this troublesome problem. Aside from injuring epithelium directly, acid causes disruption of interepithelial TJ protein complexes thereby increasing epithelial permeability[10,14,16,19]. Models assessing the mechanisms of intestinal injury have demonstrated that the critical event defining disruption of barrier function is the loss of TJ architecture and redistribution of TJ proteins such as occludin from the apical region of the interepithelial space to the cytosol[10,20-22]. In our previous work, we have demonstrated a critical role for Cl^- secretion in restoration of intestinal barrier function in ischemic-injured porcine ileum[21]. This appears to be attributable to activation of the Cl^- channel ClC-2, which is localized to the TJ[21]. More specifically, prior studies have shown that the nonselective secretory agonist prostaglandin E2 triggered rapid recovery of transepithelial electrical resistance (TER) and reduced mucosal-to-serosal fluxes of ^{3}H-mannitol in ischemic-injured intestinal mucus[20,22-24]. Previous studies have shown that activation of ClC-2 by the prostone Lubiprostone[22,25] enhanced recovery of barrier function in ischemic-injured porcine intestine. Cobiprostone (SPI-8811) is an investigational ClC-2 agonist prostone compound under development by Sucampo Pharmaceuticals Inc. (Bethesda, MD). Prostones are derived from fatty acids that are formed naturally within tissues. The present study was performed to evaluate the ability of the novel ClC-2 agonist SPI-8811 to provide gastro protection in acid-injured porcine gastric mucosa.

MATERIALS AND METHODS

Chemicals

$ZnCl_2$, Bumetanide, ^{3}H-mannitol, and cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor 172 were purchased from Sigma-Aldrich (St. Louis, MO). SPI-8811 was provided by Sucampo Pharmaceuticals Inc., Bethesda, MD.

Experimental animals

All studies were approved by the North Carolina State University Institutional Animal Care and Use committee. Yorkshire crossbred pigs of either sex approximately 10-15 kg body weight were housed individually and maintained on a commercial pelleted feed. Pigs were held off feed, but had free access to water, for 12 h before each experiment. Anaesthesia was induced with xylazine (1.5 mg/kg, im) and ketamine (11 mg/kg, im), after which they were euthanized with pentobarbital (20 mg/kg, iv). The entire stomach was clamped proximally and distally with Doyen intestinal forceps.

Ussing chamber studies

After harvesting the entire stomach, it was sharply incised at the lesser curvature and washed in porcine Ringer's (mmol/L: 154 Na^+, 6.3 K^+, 137 Cl^-, 0.3 H_2PO_4, 1.2 Ca^{2+}, 0.7 Mg^{2+}, 24 HCO_3^-, pH 7.4) and maintained in oxygenated (95% O_2/5% CO_2) Ringer's solution. The fundus portion was isolated for Ussing chamber studies. After stripping the mucosa from the seromuscular layer by using blunt scissors, mucosa was mounted in 3.14 cm^2 aperture Ussing chambers. Gastric mucosa from individual pigs was mounted on multiple Ussing chambers and subjected to acid injury and select treatments. Tissues were initially bathed in 10 mL porcine Ringer's on both mucosal and serosal sides. The serosal bathing solution contained 10 mmol/L glucose to maintain tissue viability,

and this was osmotically balanced on the mucosal side with 10 mmol/L mannitol. Indomethacin (5 μmol/L) was added on the serosal and mucosal sides of gastric tissues to prevent prostaglandin production. Bathing solutions were oxygenated and maintained at 37 ℃ by water jacketed reservoirs. The spontaneous potential difference (PD) was measured *via* Ringer's-agar bridges connected to calomel electrodes, and the PD was short-circuited *via* Ag-$AgCl_2$ electrodes using voltage clamps that corrected for fluid resistance to measure short circuit current (Isc). Transepithelial resistance ($\Omega \cdot cm^2$ was calculated from the spontaneous PD and Isc. If the spontaneous PD was between -1.0 mV and +1.0 mV, tissues were current clamped at ± 100 μA for 5 s and the PD was recorded. Isc and PD were recorded at 15 min intervals over a 90 min experimental period.

Experimental treatments

Once tissues were mounted on Ussing chambers, treatments aimed at inhibiting ClC-2 ($ZnCl_2$, 300 μmol/L, apical), CFTR (inhibitor 172, 10 μmol/L, apical), or Na(+)-K(+)-2Cl(-) cotransporter (NKCC1) (bumetanide, 100 μmol/L, basolateral) were added. Alternatively, SPI-8811 was administered to the apical surface of tissues (1 μmol/L) to enhance epithelial Cl^- secretion *via* ClC-2. Following a 30 min equilibration period, HCl (1 mol/L in Ringer's) was added to the mucosal surface of the tissue to induce acid injury. The pH was monitored with a pH meter (Hanna Instruments Inc., Ann Arbor, MI, United States) and maintained the pH at 1.5 by adding 1 mol/L HCl as needed during the experiment.

Mucosal-to-serosal fluxes of ^{3}H-mannitol

To assess mucosal permeability, 0.2 Ci/mL ^{3}H-mannitol was placed on the mucosal side of tissues after experimental treatments. After a 15 min equilibration period, standards were taken from the mucosal side of each chamber and a 30 min flux period was established by taking 0.5 mL samples from the serosal compartments. The presence of ^{3}H was established by measuring emission in a liquid scintillation counter (Rack Beta, Perkin Elmer Life and Analytical Sciences, Boston, MA, United States). Unidirectional mucosal-to-serosal ^{3}H-mannitol fluxes were calculated using a previously established spreadsheet[17].

Histological analyses

Tissue samples were collected at 0, 30 and 90 min during the experimental period and fixed in 10% formalin for histological evaluation. Paraffin embedded samples were sectioned (5 μm) and stained with hematoxylin and eosin and periodic acid-schiff stain (PAS). For each tissue, mucosal epithelial lining and gastric pits (crypts) were identified to assess the damage caused by acid (pH 1.5).

Immunofluorescence labeling of occludin

For this procedure, tissues were embedded in optimal cutting temperature medium, frozen, and sectioned at 5 μm. Tissue sections were blocked with 2% Bovine serum albumin followed by incubation with rabbit anti-occludin polyclonal antibody (1:150, Zymed, San Francisco, CA, United States) overnight at 4 ℃. Sections were washed with PBS and incubated for 45 min with FITC-conjugated anti-rabbit secondary antibody. Sections were mounted in fluorescent mounting medium, and well-orientated gastric pits were examined with a photomicroscope linked to a digital camera.

Gel electrophoresis and Western blotting

Following Ussing chamber experiments, gastric mucosal samples were snap frozen and stored at -70 ℃ prior to performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Tissue aliquots were thawed at 4 ℃ and added to chilled lysis buffer, including protease inhibitors (0.5 mmol/L Pefabloc, 0.1 mmol/L 4-nitrophenyl phosphate, 0.04 mmol/L glycerophosphate, 0.1 mmol/L Na_3VO_4, 40 μg/mL bestatin, 2 μg/mL aprotinin, 0.54 μg/mL eupeptic, and 0.7 μg/mL pepstatin A) (Sigma-Aldrich Inc., St. Louis, MO, United States) at 4 ℃. This mixture was homogenized on ice and then centrifuged at 4 ℃, and the supernatant was saved. Protein analysis of extract aliquots was performed (BCA Protein Assay Kit, Pierce, Rockford, IL, United States). Tissue extracts (amounts equalized by protein concentration) were mixed with an equal volume of 2 × SDS-PAGE sample buffers and boiled for 4 min. Lysates were loaded on an SDS polyacrylamide gradient gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a polyvinylidene fluoride membrane (Immobilon®, Millipore, Billerica, MA, United States) by using an electro blotting minitransfer apparatus. Membranes were blocked at room temperature for 2 h in Tris-buffered saline plus 0.05% tris-buffered saline tween-20 and 5% dry powdered milk, and then incubated overnight in primary antibody at 4 ℃ in rabbit anti-rat ClC-2 (Alpha Diagnostics, San Antonio, TX, United States), rabbit anti-CFTR (Santa Cruz Biotech Inc., CA, United States) or rabbit anti-occludin (Zymed Laboratories Inc., San Francisco, CA, United States). After multiple washings in TBST, membranes were incubated with horseradish peroxidase conjugated secondary antibody, and developed for visualization of protein with luminol enhancer solution (Pierce, Rockford, IL, United States).

For preparation of detergent soluble and detergent insoluble fractions of gastric mucosa, the tissue samples were extracted in lysis buffer (20 mmol/L Tris, 5 mmol/L $MgCl_2$, 0.3 mmol/L EGTA, 210 μg/mL sodium fluoride, 18.5 μg/mL sodium orthovanadate, 30 mmol/L sodium pyrophosphate, and complete mini Protease inhibitor cocktail tablet (Thermo Fisher Scientific, Rockford, IL, United States). Following brief centrifugation to remove debris, Triton X-100 soluble and insoluble fractions were collected by incubation and centrifugation (50 000 *g* for 30 min at 4 ℃) with lysis buffer containing 0.5% Triton X-100 and 0.5% SDS, respectively. The samples were processed through an SDS sample preparation kit (Ther-

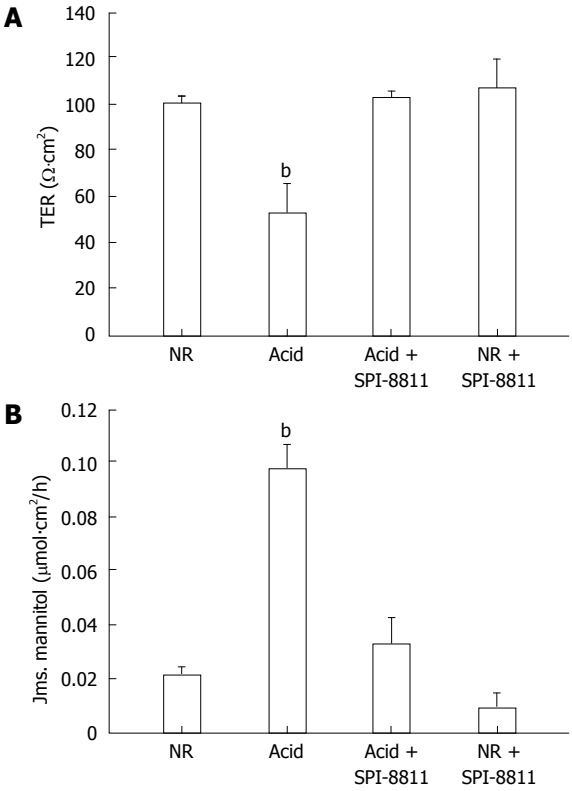


Figure 1 Barrier function of acid injured porcine gastric mucosa to pretreatment of SPI-8811 (1 μmol/L). A: Porcine gastric mucosa mounted on Ussing chambers challenged with mucosal acid (HCl, pH 1.5) over a 90 min period had significantly lower transepithelial electrical resistance (TER) (100 Ω·cm^2 *vs* 50 Ω·cm^2) when compared with control tissues bathed in normal Ringer's (NR). Mucosal application of the ClC-2 agonist SPI-8811 (1 μmol/L) prior to application of acid blocked reductions in TER (TER, 50 Ω·cm^2 *vs* 100 Ω·cm^2) .Values represent means ± SE, n = 8, $^bP < 0.01$ *vs* all other treatment groups; B: As an alternative assessment of gastric mucosal barrier function, mucosal-to-serosal fluxes of ^{3}H-mannitol were examined. In agreement with TER responses, ^{3}H-mannitol flux was significantly elevated by treatment with acid (^{3}H-mannitol flux, 0.02 μmol/L·cm^2 *vs* 0.10 μmol/L·cm^2), and this response was inhibited by SPI-8811. (^{3}H-mannitol flux, 0.10 μmol/L·cm^2 *vs* 0.04 μmol/L·cm^2). Values represent mean ± SE, n = 6, $^bP < 0.01$ *vs* all other treatment groups.

mo Fisher Scientific, Rockford, IL, United States) and a protein assay was performed before proceeding with western blotting.

Statistical analysis

Data were reported as mean ± SE. For Ussing chamber experiments, an experimental number of 6 was used. Data was analyzed by using an analysis of variance and *t*-test using a commercial statistical package (Sigmastat, Systat Software, San Jose, CA, United States). $P < 0.01$ and $P < 0.05$ were considered significant.

RESULTS

Acid injury model

To establish a gastric acid injury model, porcine gastric mucosa was mounted on Ussing chambers. After an equilibration period of 30 min the mucosal side was subjected to normal Ringer's (NR) solutions (pH 7.4) or HCl Ringer's (pH 1.5) for up to 180 min. Acid-bathed tissues had a significant decline in TER by 90 min and a further decline by 180 min (approximately 50% and 150% change in TER following 90 and 180 min of exposure to HCl, respectively, Figure 1A). Histological examination of the gastric mucosa exposed to acid for 90 min revealed injury that was limited to mucosal sloughing and partial damage to the gastric pits, resembling acute peptic ulcer disease. Alternatively, 180 min exposure to acid caused profound sloughing of the mucosal lining with damage extending into the gastric pits. Thus for further studies, gastric mucosa was exposed to HCl (pH 1.5) for 90 min to establish a more clinically relevant level of acid-induced gastric injury (data not shown).

Effects of the ClC-2 agonist SPI-8811 on TER and mucosal-to-serosal ^{3}H-mannitol fluxes in acid-injured porcine gastric mucosa

As previously described, porcine gastric mucosa was mounted on Ussing chambers and the mucosal surface was exposed to acid Ringer's (pH 1.5) for 90 min. Mucosa subjected to acid injury had significantly lower TER values when compared to the gastric mucosa bathed in NR, indicating electrophysiological evidence of disruption of epithelial barrier function. Alternatively, pretreatment of the apical side of gastric mucosa with the ClC-2 agonist SPI-8811 (1 μmol/L) nullified decreases in TER due to acid injury (Figure 1A). Epithelial permeability was assessed by mucosal-to-serosal fluxes of ^{3}H-mannitol in control and acid-injured tissues in the presence or absence of apical SPI-8811. In agreement with TER responses, acid-injured tissues exhibited increases in permeability to ^{3}H-mannitol as compared to control uninjured tissue. Pretreatment of the apical surface of mucosal tissues SPI-8811 ablated changes in permeability noted in untreated acid-injured tissues (Figure 1B).

Histological and Immunofluorescence microscopic findings in acid-injured gastric mucosa

Gastric mucosa from Ussing chambers after 90 min in NR revealed intact gastric pits with intact epithelial lining. Alternatively, when the gastric mucosa was exposed to acid (pH 1.5) over a 90 min period, extensive epithelial sloughing and erosion extending into the glandular region of the gastric mucosa and sub-epithelium was noted. Tissues pretreated with SPI-8811 had far lesser evidence of mucosal injury in response to acid, although the epithelium appeared to have flattened to maintain the barrier and the gastric pits appeared dilated (Figure 2A).

Using PAS staining for mucus we observed that the loss of mucus by acid injury was seen restored by pretreatment of ClC-2 agonist (Figure 2B). In further experiments, we performed Immunofluorescence of occludin to assess the gastric mucosal TJs. As compared to apical immunolocalization of occludin in the control uninjured gastric mucosal lining epithelium, acid-injured tissues revealed loss of occludin immunofluorescence (Figure 2C). In contrast, gastric mucosa pretreated with SPI-8811 and

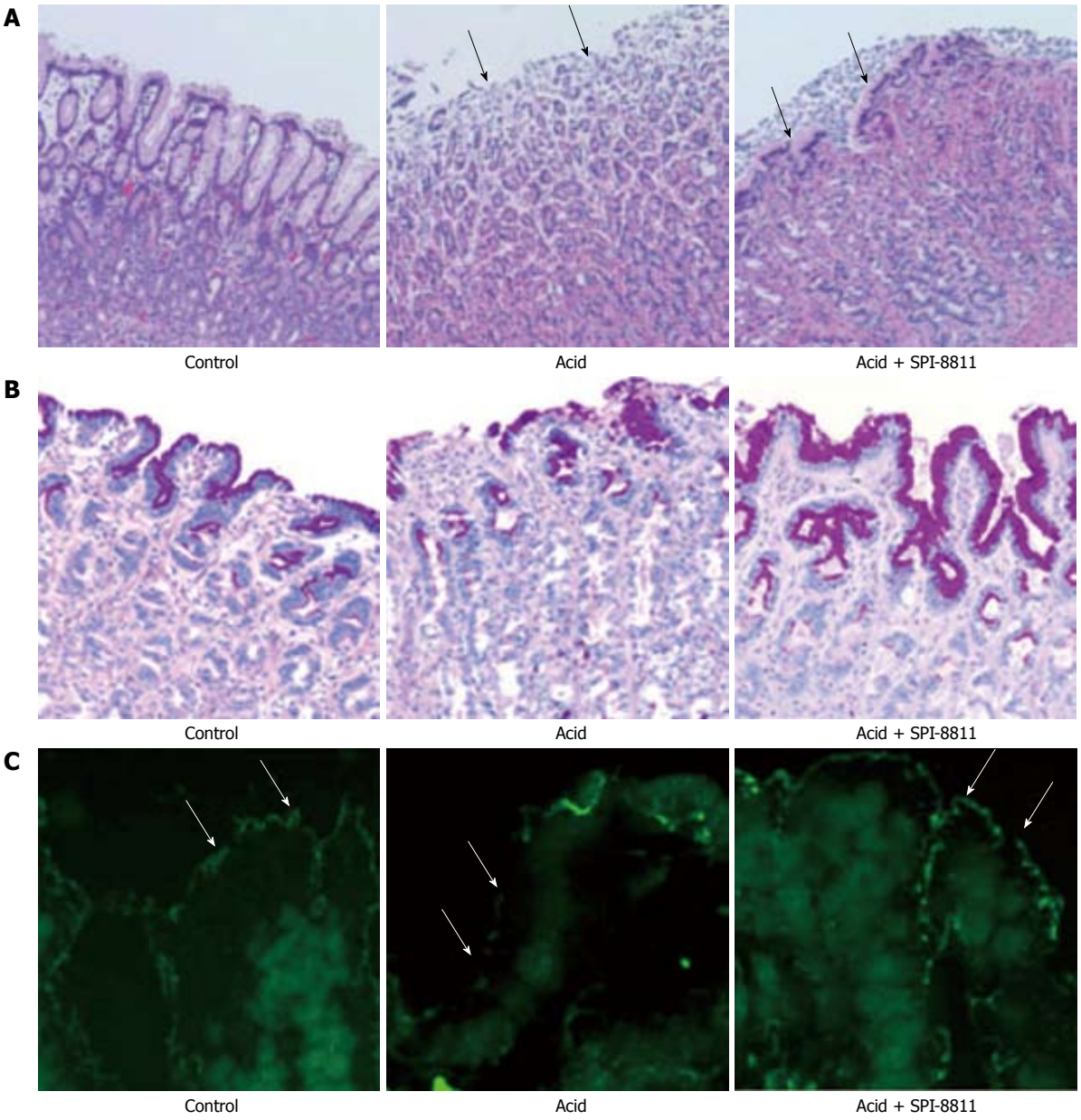


Figure 2 Histological findings in acid-injured gastric mucosal tissues. A: Tissues bathed in normal Ringer's appeared uninjured after 90 min in Ussing chambers, whereas those exposed to acid had substantial evidence of epithelial injury (× 100). Pretreatment with SPI-8811 ameliorated injury evident in acid-injured tissues. Each panel is representative of at least 3 separate animals; B: Periodic acid-schiff stain (PAS) staining findings in control tissues bathed in Ringer's solution for 90 min appeared to have normal expression of surface mucus by PAS-alcian blue staining whereas tissues exposed to acid had distinct loss to surface mucus staining (× 100). However, pretreatment with SPI-8811 prevented the loss of mucus in response to acid and thus preserved the surface mucus; C: Immunofluorescence findings for tight junction protein occludin reveals control tissues bathed in Ringer's solution for 90 min appeared to have normal immunolocalization of the tight junction protein occluding (green fluorescence) whereas tissues exposed to acid had very little visible apical epithelial occludin (× 200). However, pretreatment with SPI-8811 prevented the disorganized appearance of occludin in response to acid. Note the specific localization of occludin in each panel (arrows).

subsequently exposed to acid for 90 min had evidence of localization of occludin to the TJ.

Expression of occludin in gastric mucosal tissues

Since SPI-8811 prevented increases in paracellular permeability in response to acid, based on reduced ^{3}H-mannitol fluxes and apical epithelial localization of occludin, we evaluated expression of occludin. Occludin expression was studied in detergent soluble and detergent insoluble gastric mucosal fractions, using-actin expression as a loading control. Tissues exposed to NR had expression of multiple bands clustered at 65 kD in the detergent insoluble fraction. The finding of several bands for occludin at its expected molecular weight has been attributed to multiple occludin phosphorylation states[26]. The presence of occludin solely in the insoluble fraction was interpreted as an indication of this protein's propensity to localize to the TJ. In contrast to tissues bathed in NR, there was very little expression of occludin in acid-treated tissues, possibly because acid injury was severe enough to cause surface epithelium and interepithelial TJs. On the other hand, occludin expression in the detergent insol-

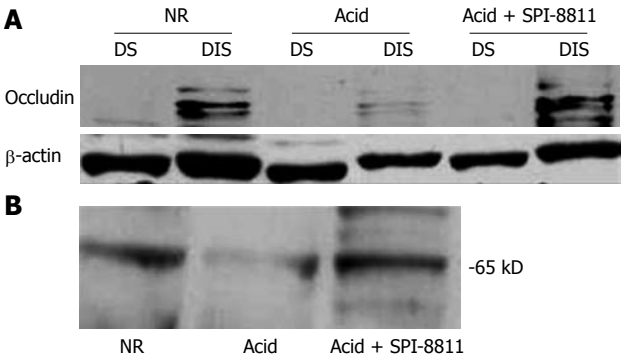


Figure 3 Expression of tight junction proteins in membrane fractions in acid injured porcine gastric mucosa. A: Western analysis revealed expression of occludin in the detergent insoluble in tissues treated with normal Ringer's (NR) alone, with very little discernable occludin in the detergent soluble fraction. On the other hand, treatment with acid (pH of 1.5 for 90 min) markedly reduced the expression of occludin in the detergent insoluble fraction, with a similar lack of occludin expression in the detergent soluble fraction. Pretreatment of tissues with mucosal SPI-8811 resulted in expression of occludin in the different fractions to a very similar extent as control tissues; B: Western analysis revealed expression of whole occludin in either control, acid injured and SPI-8811 pretreated and acid injured porcine gastric mucosa from Ussing chambers. Acid injury markedly reduced expression of whole occludin compared to control while pretreatment with SPI-8811 preserved the loss of occludin from acid injury. DS: Detergent soluble; DIS: Detergent insoluble.

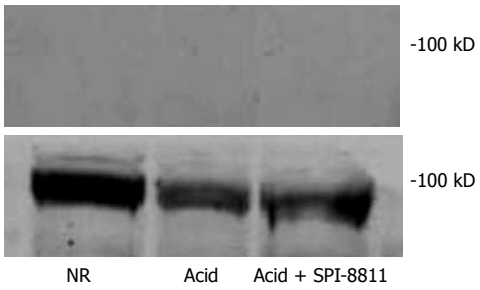


Figure 5 Expression of ClC-2 protein in porcine mucosa. Mucosal homogenates from porcine stomach were studied for expression of ClC-2 by western blotting. Expression of ClC-2 protein at its expected molecular weight (98 kD) was evident in all lanes. However, there appears to be greater expression of tissues bathed in Ringer's solution as compared to those subjected to acid or acid and SPI-8811 (n = 3). NR: Normal Ringer's.

uble fraction of tissues treated with SPI-8811 appeared similar to tissues treated with Ringer's solution alone, suggesting this prostone prevented loss of occludin. Cellular expression of occludin as a whole was also seen markedly reduced compared to control tissue and this loss was prevented with pretreatment with SPI-8811(Figure 3).

Role of Cl channels in SPI-8811-mediated protection of TER and barrier permeability in acid-injured gastric mucosa

To explore the potential role of Cl^- secretion in SPI-8811-associated gastro protection, mucosa was treated with the NKCC1 inhibitor bumetanide (100 μmol/L, basolateral) and SPI-8811 (1 μmol/L, apical), which blocked the ability of SPI-8811 to reduce acid injury (data not shown). In more targeted studies in tissues pretreated with SPI-8811, tissues were pretreated with pharmacological inhibitors of the apical chloride channels ClC-2

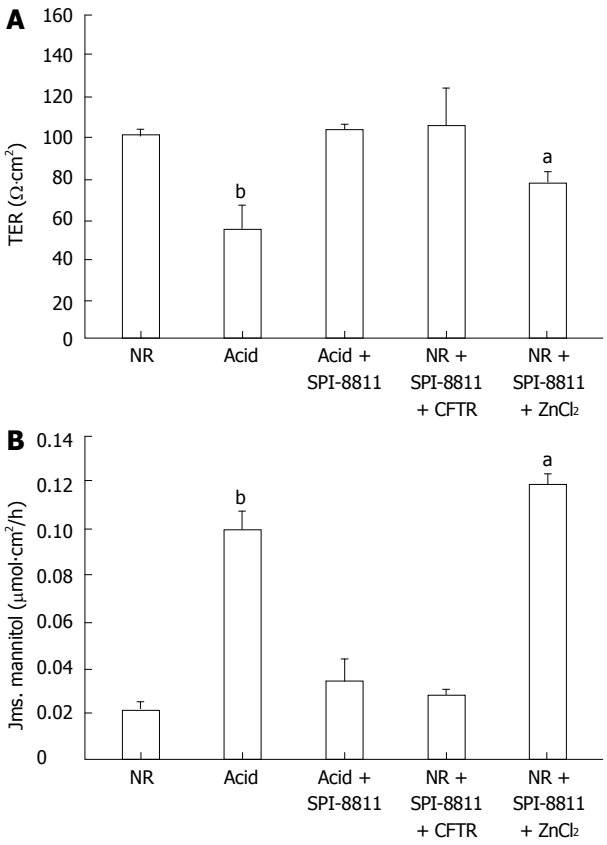


Figure 4 Electrical responses and ^{3}H-mannitol fluxes of acid-injured porcine gastric mucosa. A: Porcine gastric mucosa exposed to acid (pH 1.5 for 90 min) exhibited a significant drop in transepithelial electrical resistance (TER), which was completely blocked by pretreatment with SPI-8811. In attempt to discern which chloride channel was involved in the gastro protective mechanism of SPI-8811, ClC-2 and cystic fibrosis transmembrane conductance regulator (CFTR) were inhibited. Addition of the ClC-2 inhibitor $ZnCl_2$ ameliorated the protective effect of SPI-8811 (TER, 100 Ω·cm^2 *vs* 80 Ω·cm^2) whereas the CFTR inhibitor CFTR inhibitor 172 had no effect (TER, 100 Ω·cm^2 *vs* 100 Ω·cm^2). Values represent mean ± SE, n = 6; $^aP < 0.05$, $^bP < 0.01$ *vs* all treatment groups; B: As an alternate measure of barrier permeability mucosal-to-serosal of ^{3}H-mannitol flux were performed, and reveled a significant increase in mannitol permeability in acid injured tissues ($^bP < 0.01$). Pretreatment with SPI-8811 blocked the increase in ^{3}H-mannitol flux caused by acid injury, whereas blockade of ClC-2 inhibitor $ZnCl_2$ block the protective effect of SPI-8811 (^{3}H-mannitol flux, 0.04 μmol/L·cm^2 *vs* 0.12 μmol/L·cm^2, $^aP < 0.05$) on permeability but CFTR inhibitor had no effect on the level of protective properties of SPI-8811 on permeability (^{3}H-mannitol flux, 0.04 μmol/L·cm^2 *vs* 0.05 μmol/L·cm^2, $^bP < 0.01$). Values represent mean ± SE, n = 6. NR: Normal Ringer's.

and CFTR. Pretreatment of acid-injured mucosa with the ClC-2 inhibitor $ZnCl_2$ (300 μmol/L, apical) abolished the gastro protective properties of SPI-8811 as determined by change in TER and mannitol fluxes. On the other hand, pretreatment with CFTR inhibitor 172 (10 μmol/L, apical) had no effect (Figure 4).

Expression of ClC-2 and CFTR in porcine gastric mucosa

The literature is not clear about the expression of ClC-2 and CFTR in the porcine gastric mucosa, aside from a few studies that describe gastric mucosal changes in CFTR mouse mutants[19]. Therefore, western analyses were performed to study the expression of ClC-2 and

CFTR in porcine gastric mucosa. As shown in Figure 5, ClC-2 was expressed approximate 98 kD in porcine gastric homogenates. ClC-2 expression was observed in porcine gastric mucosa which confirmed the expression of ClC-2 in porcine stomach (Figure 5). Western analyses showed no evidence of CFTR in gastric mucosa, whereas CFTR was clearly present in porcine jejunum as a positive control (data not shown).

DISCUSSION

Prior studies have provided evidence for a critical role of ClC-2 in the recovery of mucosal barrier function in ischemic-injured intestine[20,21,27,28]. For example, Lubiprostone has been shown to interact with ClC-2 channels and enhance repair of the epithelial barrier after acute injury by ischemia[22,25,29,30]. The aim of the present study was to determine if an alternate prostone, SPI-8811, had a protective role against mucosal injury, and we chose to investigate this in the stomach. Acid plays an integral role in gastric mucosal ulceration[1,3,4,8,15,24,31]. Considering the physiological presence of acid to aid digestion in the stomach, mechanisms to provide gastro protection against injury in predisposed patients are critical. To date, this has principally included agents that ameliorate acid secretion. However, according to the results of the present study, prostones appear to provide protection in the face of low levels of pH, which would allow for continued acid secretion for normal digestive processes. Acid causes injury to the gastric mucosa in part by disrupting the TJ protein complexes, thereby increasing epithelial permeability. This in turn allows further injury by permitting permeability to luminal acid, ultimately causing erosion and then ulceration as the mucosa becomes progressively disrupted[3,14,24,32]. Among the established methods to study gastro protection and acute gastric injury are the direct application of acid or ethanol to gastric mucosa[8,31].

The first aim of our study was to produce acute acid injury resembling ulceration in clinical patients in order to study gastro protection. Accordingly, acid Ringer's solution was applied to the mucosal surface as previously described[33]. To mimic peptic ulcer disease, the body of the porcine stomach was used in this study. Optimum gastric injury was achieved (based on TER and histological examination) with a pH of 1.5, over a time period of 90 min. In each of the experiments, SPI-8811 was applied to the gastric mucosa prior to acid injury on the apical surface. Earlier studies have shown that Lubiprostone activates Cl^- secretion by a mechanism associated with recruitment of the TJ occludin in porcine intestine, thereby aiding recovery of barrier function[9,22,34,35]. Gastric mucosa was pretreated with SPI-8811 prior to induction of acid injury and showed evidence of gastro protection as evidenced by blockade of changes in TER and mannitol fluxes in tissues exposed to acid. In addition, histological changes in response to acid were ameliorated by SPI-8811. Because of the important role of TJs in maintenance of barrier function, we also examined the localization of occludin in tissues pretreated with SPI-8811 and exposed to acid. Tissues injured by acid alone appeared to have complete loss of apical epithelial occludin, possibly because of loss of cells due to severe injury, whereas tissues pretreated with SPI-8811 had evidence of apical epithelial occludin similar to that of control tissues. Further studies evaluating detergent soluble and insoluble fractions using western analyses confirmed the loss of occludin in tissues treated with acid. The reason for the loss of occludin, rather than intracellular movement of occludin, is unknown. The simplest explanation would be that the extensive reduction in pH resulted in loss of TJ proteins into the lumen, followed by erosion of epithelium and ultimately ulceration. Tissues pretreated with SPI-8811 evaluated by immunofluorescence were protected from this effect, with localization of occludin to the apical epithelium similar to that of control tissues. Additionally, occludin remained in the detergent insoluble fraction in western analyses under the influence of SPI-8811 indicating localization to the TJs.

The next aim of the study was to investigate the role of Cl^- channels in gastro protection, given that SPI-8811 is a purported Cl^- secretagogue. Because ClC-2 has been shown to be localized to interepithelial TJs in intestinal studies[22], and in gastric mucosa in the parietal cells[29,36] is a target for the prostones, we were particularly interested in this channel. The CFTR served as an alternate possibility. Our data demonstrated that SPI-8811-mediated gastro protection against acid injury was attenuated by the ClC-2 inhibitor $ZnCl_2$ whereas the selective CFTR inhibitor 172 had no effect. Although CFTR is difficult to pharmacologically inhibit, our Western analyses showed a lack of CFTR expression in porcine gastric mucosa as compared to robust expression in jejunal mucosa. In previous studies, investigators have shown that CFTR knockout mice are vulnerable to gastric ulcers due to loss of bicarbonate secretion. The lack of CFTR in porcine gastric mucosa especially in the fundus of stomach might partially explain susceptibility to acid injury in the present studies[32]. Taken together, these studies suggest that activation of ClC-2 by SPI-8811 is integral to the mechanism of gastro protection. Further studies will be required to understand the mechanisms underlying these findings.

There are opposing views regarding the role of ClC-2 in gastric chloride secretion[29,36,37] suggested involvement of ClC-2 in gastric acid secretion while another other group[38], Indicated that ClC-2 chloride secretion plays no role in production of HCl. It is also noteworthy that the apical ClC-2 channel has been suggested to serve as a route for both bicarbonate and Cl^- exit into the lumen[29,39]. This raises the possibility that ClC-2-mediated bicarbonate secretion attenuates acid injury to some extent by raising the pH. However in our studies, constant monitoring and adjustment of pH to a level of 1.5 would nullify this possibility. We also found that SPI-8811 produces a gastro protective effect not only *via* increasing gastric pH, but also by protecting and restoring the gastric mucus level (Figure 2B). The importance of gastro protection *via* mucus protection has been previously shown[7,40,34].

ClC-2 localizes to the TJ in the intestine[21,27,28,41] and this localization facilitates the interactions with TJ proteins and associated regulatory molecules. For example, recent studies have shown that ClC-2 is required for rapid reassembly of TJ proteins after ischemic injury in murine intestine[27]. The rapid process of repair is a hallmark of gastric mucosal barrier function, and may involve rapid restoration of TJs during restitution. Although this study does not completely answer questions as to the mechanism of SPI-8811, it does provide convincing evidence for the ability of SPI-8811 to protect the gastric mucosa against acid, and it does suggest this activity is at least in part attributable to a mechanism related to ClC-2 regulation.

COMMENTS

Background

Important gastric diseases such as peptic ulcer disease are aggravated by acid secretion. Injury caused by acid is characterized by damage to the gastric epithelium lining the gut. Mechanisms are in place to rapidly repair epithelial defects, and prevent epithelial barrier dysfunction and thus protect the gastric mucosa and further acid injury. Currently studies have shown the importance of the interepithelial tight junctions (TJs) in recovery of the epithelial barrier. Studies, particularly in the intestine, have shown particularly in that prostones increase the rate of epithelial recovery *via* re-assembly of TJs.

Research frontiers

The prostone lubiprostone, a new medication on the market indicated for the treatment of chronic constipation and irritable bowel syndrome has its effect on the chloride channel ClC-2. This channel is unusual in that it is located within TJs. These channels are involved in secretion of chloride in the intestine, but until now very little was about the role of ClC-2 in the stomach. Recent studies have shown that ClC-2, when activated by prostones, play an important role in re-assembly of TJs, resulting in increased rate of epithelial repair in the intestine. This study was performed to see if a prostone (cobiprostone, SPI-8811) hastened repair in the gastric mucosa *via* a mechanism involving TJs.

Innovations and breakthroughs

Activation of one of the chloride channels, ClC-2, is an innovative way to induce reassembly of TJs during repair of the epithelial mucosal barrier. Gastric epithelium is continuously exposed to gastric acid and in the case of peptic ulcer disease the gastric acid secretion disrupts the barrier and thus aggravates the condition. In such cases, reducing acid secretion by using various acid secretory antagonists is the most common way to prevent gastric injury. Other alternatives to prevent breakdown of the gastric barrier function are limited. The present data show an innovative breakthrough in the treatment of acid induced gastric epithelium. The authors' findings were consistent with prior studies showing enhanced repair with prostones in the intestine at the level of TJs.

Applications

By understanding the role of prostone activation of ClC-2 in acid injured gastric porcine mucosa, this study might represents a future strategy to therapeutic intervention in patients with non-steroidal anti-inflammatory drugs injury and peptic ulcer disease. Further basic science research followed by clinical trials will be needed to determine the validity of these findings.

Terminology

The term ClC-2 is used to describe a chloride channel in the gut epithelium that is localized to interepithelial TJs. The term prostone refers to a new group of compounds which are distinct from prostaglandins and specifically activate ClC-2.

Peer review

The present study was performed using porcine tissues; it demonstrates that the novel ClC-2 agonist SPI-8811 stimulates recovery of barrier function in acid injured porcine gastric mucosa by preventing loss of the TJ protein occludin. The data were appropriately analyzed and indicate SPI-8811 has an impact on protecting epithelial barrier function by preventing breakdown of TJs and thus prevent further deep injury. Additional molecular studies need to be performed to understand how ClC-2 interacts with prostones to protect the epithelial TJs.

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ORIGINAL ARTICLE

Expression of the P2X$_2$ receptor in different classes of ileum myenteric neurons in the female obese *ob/ob* mouse

Márcia Sanae Mizuno, Amanda Rabello Crisma, Primavera Borelli, Patricia Castelucci

Márcia Sanae Mizuno, Patricia Castelucci, Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo, CEP São Paulo 05508-900, Brazil
Amanda Rabello Crisma, Primavera Borelli, Faculty of Pharmaceutical Sciences, University of São Paulo, CEP São Paulo 05508-900, Brazil
Author contributions: Mizuno MS performed and evaluated the immunohistochemistry; Crisma AR and Borelli P were involved in blood analyses; and Castelucci P designed the study, wrote and edited the manuscript.
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Correspondence to: Dr. Patricia Castelucci, PhD, Assistant Professor, Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Dr. Lineu Prestes, 2415, CEP São Paulo 05508-900, Brazil. pcastel@usp.br
Telephone: +55-11-30918463 Fax: +55-11-30917366
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Abstract

AIM: To examine whether the *ob/ob* mouse model of obesity is accompanied by enteric nervous system abnormalities such as altered motility.

METHODS: The study examined the distribution of the P2X$_2$ receptor (P2X$_2$R) in myenteric neurons of female *ob/ob* mice. Specifically, we used immunohistochemistry to analyze the co-expression of the P2X$_2$R with neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), and calretinin (CalR) in neurons of the small intestine myenteric plexus in *ob/ob* and control female mice. In these sections, we used scanning confocal microscopy to analyze the co-localization of these markers as well as the neuronal density (cm^2) and area profile (μm²) of P2X$_2$R-positive neurons. In addition, enteric neurons were labeled using the nicotinamide adenine dinucleotide (NADH) diaphorase method and analyzed with light microscopy as an alternate means by which to analyze neuronal density and area.

RESULTS: In the present study, we observed a 29.6% increase in the body weight of the *ob/ob* animals (OG) compared to the control group (CG). In addition, the average small intestine area was increased by approximately 29.6% in the OG compared to the CG. Immunoreactivity (IR) for the P2X$_2$R, nNOS, ChAT and CalR was detectable in the myenteric plexus, as well as in the smooth muscle, in both groups. This IR appeared to be mainly cytoplasmic and was also associated with the cell membrane of the myenteric plexus neurons, where it outlined the neuronal cell bodies and their processes. P2X$_2$R-IR was observed to co-localize 100% with that for nNOS, ChAT and CalR in neurons of both groups. In the *ob/ob* group, however, we observed that the neuronal density (neuron/cm^2) of P2X$_2$R-IR cells was increased by 62% compared to CG, while that of NOS-IR and ChAT-IR neurons was reduced by 49% and 57%, respectively, compared to control mice. The neuronal density of CalR-IR neurons was not different between the groups. Morphometric studies further demonstrated that the cell body profile area (μm²) of nNOS-IR, ChAT-IR and CalR-IR neurons was increased by 34%, 20% and 55%, respectively, in the OG compared to controls. Staining for NADH diaphorase activity is widely used to detect alterations in the enteric nervous system; however, our qualitative examination of NADH-diaphorase positive neurons in the myenteric ganglia revealed an overall similarity between the two groups.

CONCLUSION: We demonstrate increases in P2X$_2$R expression and alterations in nNOS, ChAT and CalR IR in ileal myenteric neurons of female *ob/ob* mice compared to wild-type controls.

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Key words: Obesity; P2X$_2$ receptor; Myenteric neurons; Chemical coding; Mouse

Peer reviewers: Romeu R Souza, Professor, Department of Morphology, Laboratorio de Biodinamica, Universidade Sao Judas Tadeu, Rua Afonso de Freitas, 451 ap 122, São Paulo

04006052, Brazil; Dr. Maria Raquel Marçal Natali, Department of Morphology Science, State University of Maringá, Av, Colombo 5790, Maringá 87020-900, Brazil

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INTRODUCTION

Obesity is a chronic heterogeneous disorder characterized by abnormal or excessive fat accumulation that presents a risk to health[1]. The leptin-deficient *ob/ob* mouse exhibits both obesity and peripheral diabetic neuropathy, traits characteristic of human patients, and thus represents a valuable animal model of obesity and type 2 diabetes-like pathology[2].

Obesity is associated with an increased risk of type 2 diabetes and is often accompanied by complications of the gastrointestinal tract such as gastroparesis, constipation, diarrhea, and fecal incontinence[1]. Disruptions in the function of the gastrointestinal tract have been associated with alterations in the enteric nervous system[3]. Enteric neurons comprise both the myenteric and submucosal plexuses, which control the motility of the intestine, the transport of fluids from the intestinal mucosa, and local blood flow[3]. The groups of neuronal cell bodies (ganglia) in these plexuses are interconnected by nerve fiber bundles[3]. The myenteric and submucosal plexuses also regulate gastrointestinal secretions important for the digestion of food particles, intestinal lubrication, nutrient uptake, regulation of pH, and the concentration of solutes and elimination of waste products[3]. Studies of chemical coding in neurons of the mouse enteric nervous system have been particularly important to our understanding of the function of enteric neurons[4,5]. Defects in enteric nervous system function have been reported in *ob/ob* mice, including alterations in intestinal motility[6] and in the enteric neurons[7,8]. Down-regulations in intestinal motility have been reported in obese humans[1]. Thus, the mouse is a valuable animal model for the study of obesity-related pathology in the enteric nervous system.

Adenosine 5'-triphosphate (ATP) has been well-established as a neurotransmitter and a ligand of the P2X receptor family, which is made up of seven known receptor subunits (PX_{1-7})[9]. These receptors play an important role in synaptic transmission within the neural pathways that mediate intestinal motility[10]. Previous immunohistochemical studies have documented the distribution of $P2X_2$ receptors ($P2X_2Rs$) in the enteric nervous system of the guinea pig[11-14], rat[15-18] and mouse[19-21]. However, the distribution of $P2X_2R$ expression in the enteric nervous system of obese mice remains unstudied.

It has been reported that the $P2X_2R$ is expressed in inhibitory, cholinergic, and intrinsic afferent neurons in the enteric nervous system[11]. The goal of the current study is to analyze the chemical coding, density, and area profile of neuron immunoreactivity (IR) for the $P2X_2R$, neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), calretinin (CalR), and positivity for nicotinamide adenine dinucleotide (NADH)-diaphorase activity in the small intestine enteric nervous system of female *ob/ob* and control mice.

MATERIALS AND METHODS

Animals

Two groups of 11-mo-old female mice were compared in these studies. The obese group (OG) consisted of six homozygous (*ob/ob*) C57BL/6J mice, and the control group (CG) was comprised of six wild-type C57BL/6J mice. The animals were bred at the State University of Campinas Breeding Center. The animals were housed at five per cage in an artificially lit room (12 h/12 h, light/dark) and were fed a standard pellet diet (Nuvilab, São Paulo, Brazil) and water *ad libitum*. Animals were euthanized for experiments using a CO_2 chamber. This study was conducted according to current legislation on animal experiments at the Biomedical Science Institute of the University of São Paulo.

Collection of blood for biochemical analysis

Samples of blood were obtained *via* the puncture of the plexus axillary artery of mice previously anesthetized with xylazine (16 mg/kg) and ketamine (120 mg/kg). Following collection, the blood remained at rest for 2 h at room temperature for clot formation, and then was centrifuged at 2500 × *g* for 20 min at 4 ℃ to obtain the serum. The serum supernatant was maintained at -40 ℃ until being analyzed for glucose composition.

Immunohistochemistry

Following CO_2 euthanasia, fresh segments of distal ileum were removed from each animal and placed in phosphate-buffered saline (PBS: 0.15 mol/L NaCl in 0.01 mol/L sodium phosphate buffer, pH 7.2) containing nicardipine (10^{-6} mol/L; Sigma, United States) to inhibit tissue contraction. The dissected segments were sliced open along the mesenteric border and cleaned of their contents using PBS. They were then pinned out tautly, mucosa side down, onto a balsa-wood board and fixed overnight at 4 ℃ in paraformaldehyde in 0.2 mol/L sodium phosphate buffer (pH 7.3). The next day, the segments of ileum tissue were cleared of fixative with three 10-min washes in 100% dimethyl sulfoxide followed by three 10-min washes in PBS. All tissue was stored at 4 ℃ in PBS containing sodium azide (0.1%). The fixed tissue was then dissected and the mucosal, submucosal, and circular layers were removed to obtain whole-mounts of the longitudinal muscle-myenteric plexus. In a second type of preparation, the mucosa and muscularis externa were removed to leave the intact submucosal layer. Whole-mount preparations of the myenteric and submucosa of the ileum were then pre-incubated in blocking buffer, 10% normal horse

Table 1 Characteristics of primary and secondary antibodies

Antigen	Host	Dilution	Code and reference
$P2X_2$ receptor	Rabbit	1:120	AB5244, Chemicon
Nitric oxide synthase	Sheep	1:2000	H205 (Williamson *et al*[31], 1996)
Choline acetyltransferase	Goat	1:50	Chemicon
Calretinin	Goat	1:100	CG1 (Swant)
Secondary antibodies			
Donkey anti-rabbit IgG Alexa 488	Donkey	1:500	Molecular probes
Donkey anti-sheep IgG Alexa 594	Donkey	1:100	Molecular probes

serum in PBS containing 1.5% Triton X-100 for 45 min at room temperature to reduce non-specific antibody binding and to permeabilize the tissue, respectively (Table 1). To localize $P2X_2R$ IR, we used a rabbit antiserum raised against amino acids 457-472 of the rat $P2X_2R$, with a single Cys extension at the N-terminal (AB5244 from Chemicon, Temecula, CA, United States). Tissues were incubated with the antibody at a dilution of 1:120 in blocking buffer for 48 h at 4 ℃. Dual immunohistochemistry was achieved using combinations of antisera (Table 1). Following incubation in primary antisera, the tissue samples received three 10-min washes in PBS and were then incubated in a mixture of secondary antibodies (Table 1). Following three further 10-min washes in PBS, the tissue samples were mounted onto microscope slides in glycerol buffered with 0.5 mol/L sodium carbonate buffer (pH 8.6). Tissues preparations were examined using both Leica and Nikon epifluorescent microscopes. Images were captured using a digital camera coupled to Image-Pro Plus software. Preparations were also analyzed by confocal microscopy using a Zeiss confocal scanning laser system installed on a Zeiss Axioplan 2 microscope. The image dimensions were 512 × 512 pixels, with an optical section thickness of 0.5 μm. Immunoreactive cells were scanned as a series of optical sections, with a center spacing of 0.2 μm, and the images were collected using LSM 5 Image Zeiss processing software, and further processed using Corel Photo Paint and Corel Draw software programs.

Statistical analysis

Antigen colocalization was determined by examining preparations immunolabeled with two fluorescent secondary antibodies. Neurons of interest were identified by immunofluorescence for one antigen; the filter was switched, and labeling for the second antigen was determined. In this way, the proportion of neurons immunoreactive for both antigens was determined. For each antigen pair, the cohort size was 100 neurons. Co-IR for the $P2X_2R$ with that of the other antigens (nNOS, ChAT, or CalR) was assessed. The percentage of double-immunoreactive neurons was calculated and expressed as mean ± SE (n = number of tissue preparations). The number of neurons immunoreactive for the $P2X_2R$, nNOS, CalR, and ChAT, as well as the neuronal profile (area of cell body), were measured by examining the whole-mount preparations under a binocular microscope at 100 × magnification. All neurons present in each cm^2 were counted. The profile areas of 50 nerve cell perikarya from each animal were measured using Image-Pro Plus software. Data were compared by one-way analysis of variance (ANOVA) and Student's t-tests; $P < 0.05$ was considered to be statistically significant.

NADH diaphorase staining

A separate cohort of 12 animals was weighed and euthanized in a CO_2 chamber prior to the opening of the anterior abdominal wall. The distal ileum was removed and washed in Krebs solution. The surface area of the entire small intestine was measured using a planimeter. Each piece of ileum was then ligated with cotton thread at the proximal end and gently distended with Krebs solution introduced with a syringe into the distal end. When the intestine was sufficiently distended, the syringe needle was withdrawn and the ligature was simultaneously tightened. The following steps were then performed to label neurons using the NADH diaphorase histochemical technique[22,23]. Following incubation in Krebs solution at room temperature for 15-30 min, the small intestine was transferred to a permeabilizing agent (0.3% Triton-X 100 in Krebs solution) for 15-90 s and then submitted to three 10-min washes in Krebs solution. The specimens were incubated for 30-45 min at 20 ℃ in 20 mL of incubation medium that contained 0.5 mg/mL of nitro blue tetrazolium (Sigma Chemical Co, St. Louis, MO) in distilled water (25 parts), 0.1 mol/L sodium phosphate buffer, pH 7.3 (25 parts), distilled water (50 parts), and 0.5 mg/mL of the reduced form of β-NADH (Sigma, United States). The reaction was stopped by immersion in 10% buffered formalin solution, in which the tissue samples were fixed for a minimum of 24 h at room temperature. Whole mount preparations were then prepared as follows: the small intestine was opened, the mucosa was removed, and the longitudinal muscle, with the myenteric plexus attached, was lifted at one corner and gently removed from the entire strip. After several washes in distilled water, fragments (2 cm^2) of the proximal, middle, and distal portions of the large intestine were prepared as whole mounts in glycerol on microscope slides and sealed with Entellan (Merck).

Myenteric neurons were identified by the presence of intense formazan reaction product filling the perikaryon, as well as by large, round, and unstained nuclei. The number of neurons and the profile areas of the nerve cell bodies were measured by examining the whole mount preparations under a binocular microscope at a magnification of 400 ×. All labeled neurons in each fragment were counted. The profiles of 100 nerve cell perikarya from each portion were obtained using a semi-automatic morphometry device (Image-Pro Plus Program 3.1)[17,18].

Results are expressed as mean ± SE. Data were compared by ANOVA with Student's t-tests for multiple comparisons, as appropriate. $P < 0.05$ was considered statistically significant.

Table 2 Body weight, small intestine area and glucose levels in female control (n = 4) and *ob/ob* mice (n = 4)

	Control group	Obese group
Body weight (g)	25.62 ± 0.62	65.52 ± 1.08[a]
Small intestine area (cm^2)	26.6 ± 2.8	34.5 ± 3.21[a]
Glucose (mg/dL)	195.75 ± 58.09	322.2 ± 99.2[a]

Student's *t*-tests for multiple comparisons. [a]$P < 0.05$ *vs* control.

RESULTS

In the present study, we found a significant (29.6%, $P < 0.05$) increase in the body weight of the *ob/ob* animals (OG) compared to controls (Table 2). In addition, the average small intestine area was increased by approximately 29.6% in the OG as compared to the CG ($P < 0.05$; Table 2). Plasma glucose measurements revealed levels of 195.75 ± 58 mg/dL in control mice and 322.3 ± 58 mg/dL in the OG mice ($P < 0.05$). Mice with blood glucose levels above 300 mg/dL were considered to be diabetic (Table 2).

Immunolabeling

IR for the $P2X_2R$, nNOS, ChAT and CalR was detectable in the myenteric plexus, as well as in the smooth muscle, in both groups. This IR mainly occurred throughout the cytoplasm and on cell surface and nuclear membranes, and effectively outlined the neuronal cell bodies and their processes in the myenteric plexus (Figure 1). nNOS-IR neurons were detected in both groups. Some nNOS-IR neurons displayed short dendritic processes, characteristic of Dogiel type I morphology. The primary and secondary fiber tracts were also immunoreactive for nNOS (Figure 1). ChAT-IR neuronal cell bodies were abundant in the myenteric ganglia; ChAT-IR was present in the cytoplasm of neurons from both groups. Nearly all of the ChAT-IR neurons displayed Dogiel type I morphology. We also observed a small number of large, smooth-surfaced ChAT-IR cell bodies. In addition, varicose ChAT-IR fibers were observed surrounding the ganglia, and constituted the primary and secondary fiber tracts of the ileum myenteric plexus (Figure 2). IR for CalR was detected in neuronal cell bodies and axonal/dendritic processes in both the control and the OG. Some neurons showed typical Dogiel type I morphology, with short dendritic processes, while others displayed long axonal projections characteristic of Dogiel type Ⅱ morphology (Figure 2).

Statistical analysis

Double immunolabeling for the $P2X_2R$ in combination with anti-nNOS, ChAT, and CalR IR was conducted to identify different chemical classes (chemical coding) of neurons in the myenteric plexus. In the CG, 99.5% ± 0.6% of NOS-IR neurons exhibited $P2X_2R$-IR and, in the OG, the colocalization score was similar (99.1% ± 1.1%). The colocalization of ChAT-IR and CalR-IR with $P2X_2$-IR was 100% in neurons from both the control and the OGs (Table 3). In summary, these analyses did not show any statistically significant differences in the colocalization of $P2X_2R$-IR with that of the other antigens between the two groups (Table 3).

Table 3 Results of the quantitative analysis of double labeling studies of immunoreactivity against the $P2X_2$ receptor, neuronal nitric oxide synthase, choline acetyltransferase, and calretinin in the myenteric plexus of the ileum from control and *ob/ob* female mice (n = 4, mean ± SE) (%)

	Control group	Obese group
$nNOS\text{-}IR^+/P2X_2\text{-}IR^+$	99.5 ± 0.6	99 ± 1.0
$ChAT\text{-}IR^+/P2X_2\text{-}IR^+$	100	100
$CalR\text{-}IR^+/P2X_2\text{-}IR^+$	100	100
$P2X_2\text{-}IR^+/nNOS\text{-}IR^+$	19.5 ± 3.6	19.5 ± 3.6
$P2X_2\text{-}IR^+/ChAT\text{-}IR^+$	23.6 ± 4.2	24.6 ± 4.37
$P2X_2\text{-}IR^+/CalR\text{-}IR^+$	23.7 ± 3.75	21.6 ± 1.20

nNOS: Neuronal nitric oxide synthase; ChAT: Choline acetyltransferase; CalR: Calretinin; IR: Immunoreactivity.

Upon examination of neuronal density in the myenteric plexus, we found an increase of 34.3% in the density of $P2X_2R$-IR neurons in the OG compared to controls ($P < 0.05$). In addition, the density of nNOS-IR and ChAT-IR neurons decreased by 42.6% and 53.4%, respectively, in the OG compared to controls ($P < 0.05$). There were no differences between the groups in the density of CalR-IR neurons (Figure 3). These data suggest that the density of $P2X_2R$-IR neurons increases, while that of nNOS-IR and ChAT-IR neurons decreases, in the OG compared to controls. We then estimated the total number of neuron IR for the $P2X_2R$ and found an increase of 105% in the OG compared to controls, while the total number of nNOS-IR and ChAT-IR neurons was decreased 35% and 47%, respectively, compared to controls ($P < 0.05$). The total number of CalR-IR neurons was not significantly different between the two groups (Figure 3).

We then evaluated the number of $P2X_2R$-IR neurons per myenteric ganglion in both groups. The number of $P2X_2$-IR neurons was 19 ± 1.8 neurons/ganglion in the OG *vs* 18.6 ± 3.9 neurons/ganglion in the CG. In addition, the number of nNOS-IR neurons per ganglion did not significantly differ between the groups, and was 6.6 ± 1.4 neurons/ganglion in the CG, and 5.2 ± 0.7 neurons/ganglion in the OG. The number of ChAT-IR neurons per ganglion also did not differ between the groups (5.9 ± 1.8 for CG, 5.1 ± 1.4 for OG). The number of CalR-IR neurons per ganglion also did not differ between groups (6.5 ± 1.2 for CG, 5.0 ± 0.2 for OG). Therefore, these data show that there were no significant differences between the control and OGs in the numbers of $P2X_2R$-, nNOS-, ChAT- or CalR-IR neurons in the myenteric ganglia (Figure 3).

Neurons with typical Dogiel type I and Ⅱ morphologies were observed in whole mounts immunolabeled for CalR-IR. The density of Dogiel type I neurons in the CG and OG was 6.3 ± 1.4 neurons/cm^2 and 5.7 ± 0.3

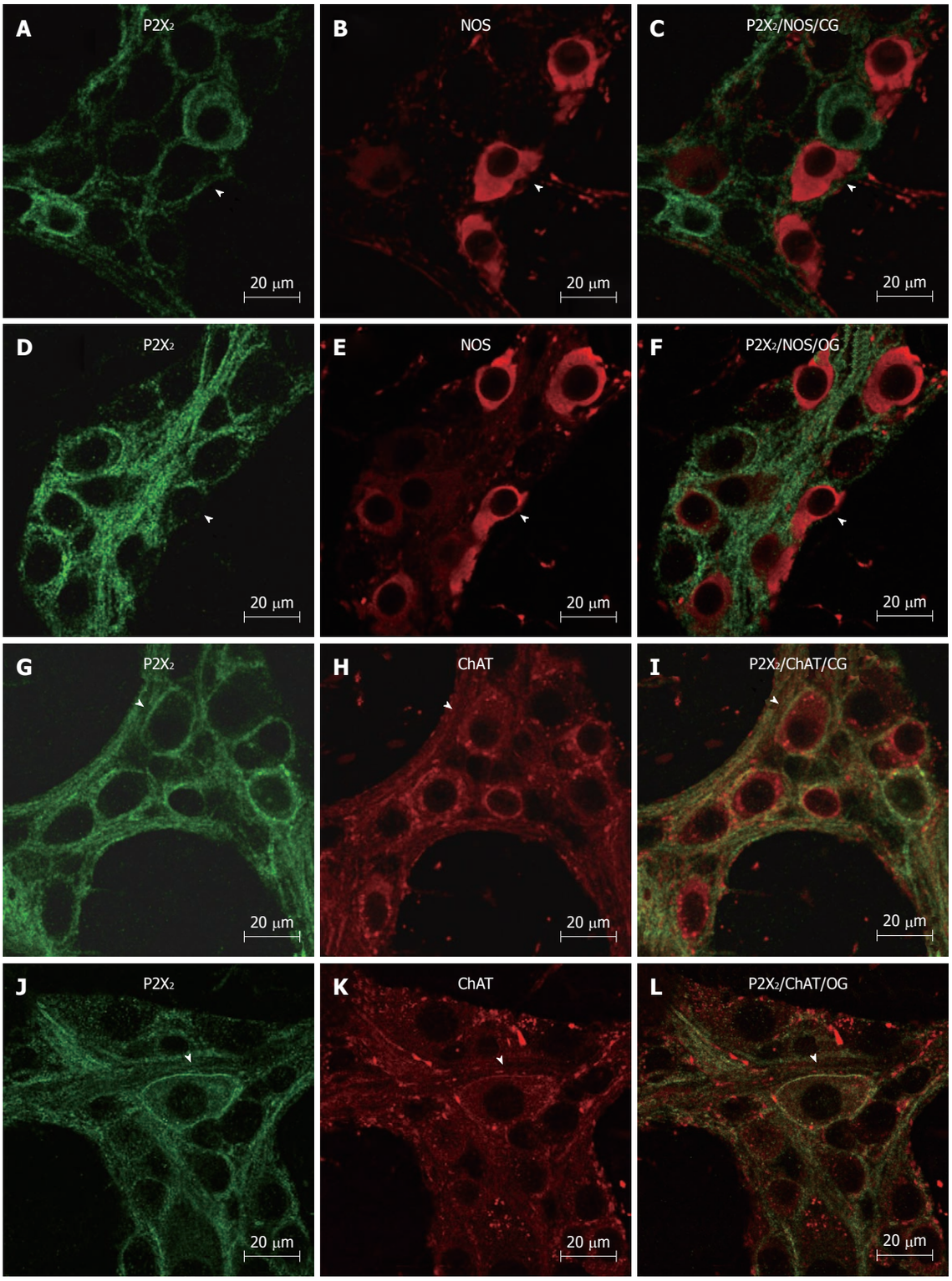


Figure 1 Immunoreactivity for $P2X_2$ receptor (A, D, G, J), nitric oxide synthase (B, E), choline acetyltransferase (H, K; merged images C, F, I, L) was examined in ileum myenteric plexus neurons from the control group (A-C, G-I) and the obese *ob/ob* group (D-F, J-L). Single arrows show double-labeled $P2X_2$ receptor ($P2X_2R$)- and nitric oxide synthase (NOS)-immunoreactivity neurons in the control group (CG) (A-C) and obese group (OG) (D-F) groups. Double-labeling for $P2X_2R$ and choline acetyltransferase (ChAT) in CG neurons (G-I) and OG (J-L) is indicated by arrowheads.

neurons/cm^2, respectively. The density of Dogiel type Ⅱ neurons was 1.3 ± 0.2 neurons/cm^2 in the CG and 1.5 ± 0.2 neurons/cm^2 in the OG. These data show that there were no significant differences in the density of CalR-IR Dogiel type Ⅰ and Ⅱ neurons between the CG and OG (Figure 3).

We then measured the cell body area of neurons in the myenteric plexus from both control and OGs.

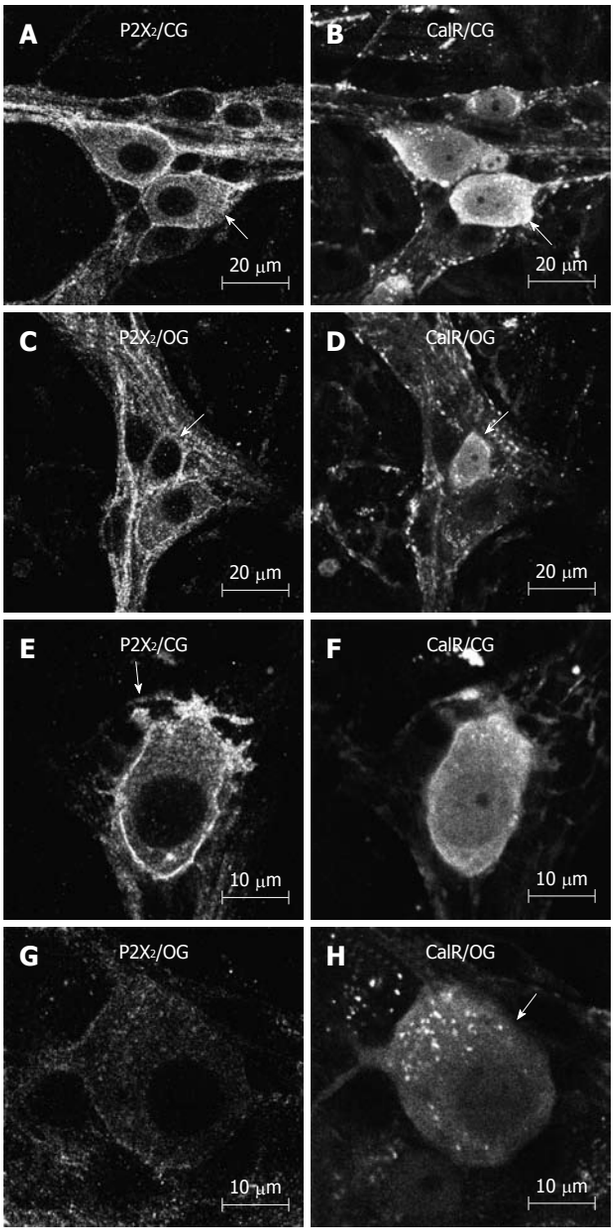


Figure 2 Immunoreactivity for P2X2 receptor (A, C, E, G) and calretinin (B, D, F, H) was examined in the ileum myenteric neurons of the control group (A, B, E, F) and the obese group (C, D, G, H). Single arrows show double labeling for P2X2 receptor and calretinin (CalR) in the neurons of the control group (CG) (A, B) and the obese group (OG) (C, D). E, F: **An example of a Dogiel type Ⅰ neuron with many lamellar and short dendrites; G, H: A neuron with** Dogiel type Ⅱ morphology with a round and smooth cell body and lipofuscin evident in the cytoplasm (arrow).

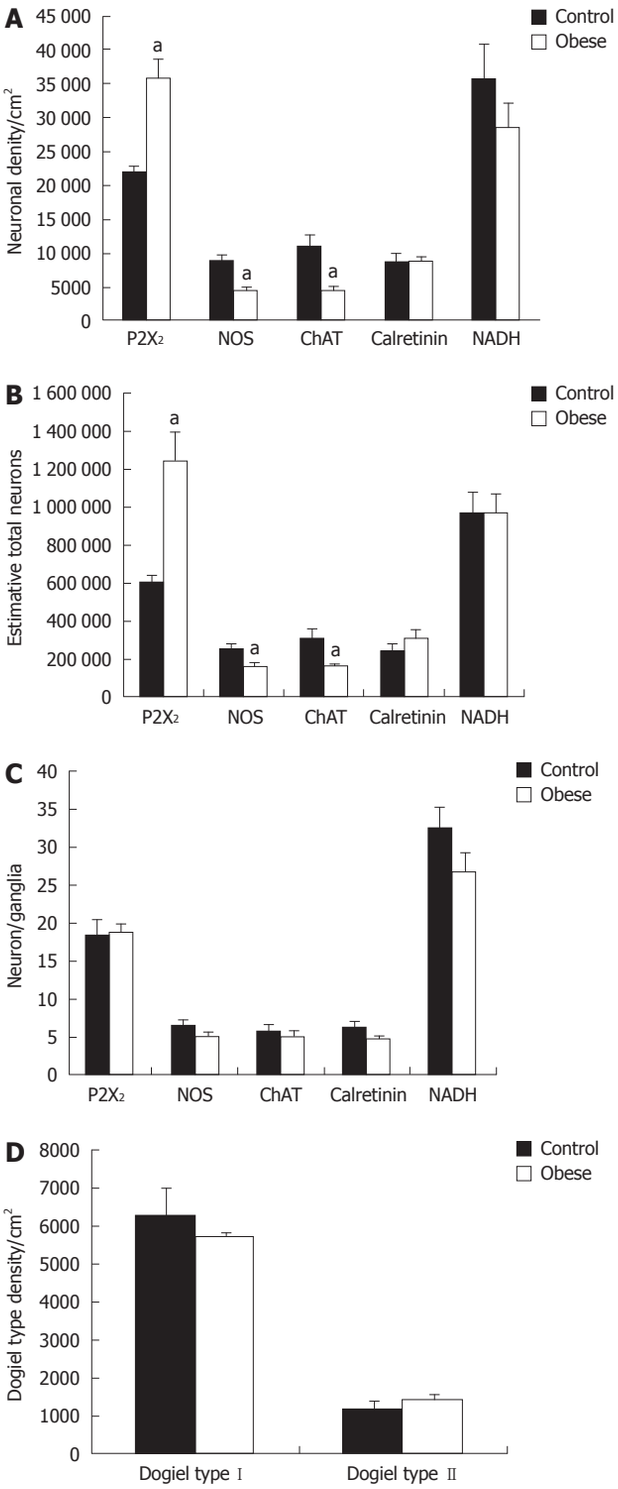


Figure 3 Neuronal density (neurons/cm^2) (A), the estimated total neuron number (B) and neurons per ganglion immunoreactive for P2X2 receptor, nitric oxide synthase, choline acetyltransferase, calretinin, and positive for nicotinamide adenine dinucleotide-diaphorase activity in the ileum myenteric plexus of control and obese mice (C), and densities of Dogiel type Ⅰ and Ⅱ neurons immunoreactive for calretinin (D) in control and obese mice. $^aP < 0.05$ *vs* control, mean ± SE, Student's *t*-test. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase; NADH: Nicotinamide adenine dinucleotide.

Although there was no change in the size of P2X2R-IR neurons between the groups, we observed a significant increase in the cell body areas of nNOS-IR (34%), ChAT-IR (17%), and CalR-IR neurons (35%) in the OG compared to controls ($P < 0.05$; Figure 4). The size distribution of neurons in the myenteric plexus of CG and OG mice are shown in Figure 5.

NADH-diaphorase analysis

Our qualitative examination of NADH-diaphorase positive neurons in the myenteric ganglia revealed an overall similarity between the two groups. With this method, the formazan reaction product labels the neuronal cy-

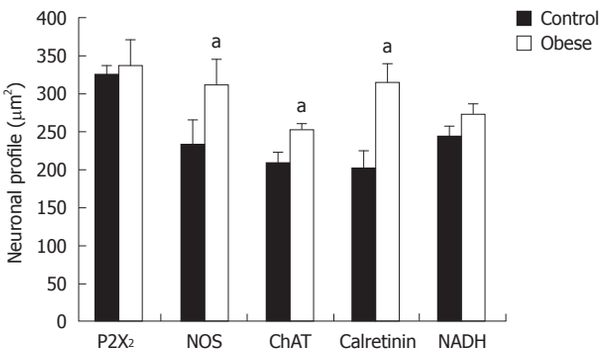


Figure 4 Cell body profile areas (μm²) of neurons immunoreactive for P2X2 receptor, nitric oxide synthase, choline acetyltransferase, calretinin and nicotinamide adenine dinucleotide-diaphorase in the ileum myenteric plexus of control and obese groups. $^{a}P < 0.05$ *vs* control, mean ± SE, Student's *t*-test. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase; NADH: Nicotinamide adenine dinucleotide.

toplasm with a varying intensity, while neuronal nuclei appear translucent. We did observe that the myenteric neurons appeared to be more widely-spaced (less dense, or grouped) in the myenteric ganglia of the OB group compared to controls (Figure 6).

This histochemical analysis provided some potentially useful general information about the myenteric neuronal population in the mouse ileum. The density of NADH-diaphorase-positive neurons was reduced by about 20% in the OG compared to controls; however, this reduction was not statistically significant (Figure 3). The total surface area of the small intestine in both groups was measured, and the total number of neurons was estimated to be 971 558 ± 205 000 in the CG and 974 042 ± 187 000 in the OG (Figure 3). In addition, an estimate of the number of neurons per myenteric ganglion did not reveal any significant differences between the groups (Figure 3). Cell body area profile measurements of NADH-diaphorase-positive neurons also showed no differences in overall neuronal size between the control and OGs (Figure 4).

DISCUSSION

Gastrointestinal disorders arising from alterations in intestinal motility in obese individuals have been related to alterations in the enteric nervous system[1]. In the current report, we demonstrate changes in the population of enteric neurons of the myenteric plexus in the *ob/ob* mouse that may explain, in part, impairments in intestinal motility suffered by obese individuals[1]. The current study demonstrates, for the first time, the presence of P2X2R IR in the myenteric plexus, and alterations in the chemical identity, or coding, of myenteric neurons in the ileum of obese female *ob/ob* mice compared to controls. Previous studies have revealed the presence of P2X2,3,7 receptor-containing neurons in the enteric nervous systems of various species[11-21]. In the current study, we show that IR for the P2X2R is present in both the cytoplasm and membrane of myenteric plexus neurons in control and obese animals. We confirm that IR for the P2X2R colocalizes with that for nNOS, ChAT and CalR in myenteric neurons. This finding is consistent with previous work demonstrating the presence of P2X2R in inhibitory neurons, as well as in intrinsic excitatory and secretomotor/vasodilator primary afferent neurons in the guinea pig[11] and rat[16-18]. We did not observe any differences in the co-localization of P2X2R with IR for these markers (nNOS, ChAT, CalR) between the control and obese mice, suggesting that the neurochemical coding of these neurons is not altered in the myenteric plexus of obese female *ob/ob* female mice compared to wild-type controls.

The creation of the *ob/ob* mouse on the C57BL/6J background, which followed the cloning and sequencing of the mouse *ob* gene and its human homologue, provides an excellent model for the study of obesity[2]. These mice present with obesity that is accompanied by the manifestation of motor and sensory nerve conduction deficits, small sensory nerve fiber neuropathy, intraepidermal sensory nerve fiber loss, and oxidative nitrosative stress in peripheral nerves, features that are characteristic of human subjects with obesity and diabetes[2]. Previous studies have demonstrated that mice with blood glucose levels above 300 mg/dL are useful as a mouse model of diabetes[2]. In the current study, the obese *ob/ob* mice had average blood glucose levels of 322.3 ± 58 mg/dL, clearly within the diabetic range. Consistent with previous reports, the body weights of the *ob/ob* mice were twice those of the control mice in our study. This increase in body weight was accompanied by a similar increase in the area of the ileum in the obese animals, in accordance with previous findings[6]. Obesity in humans is associated with increased risk of type 2 diabetes and is often accompanied by complications of the gastrointestinal tract. In the current work, we found high levels of glucose, well within the diabetic range, accompanied by alterations in the myenteric neurons in the obese *ob/ob* mice compared to controls, providing a possible explanation for the dysfunctions in intestinal motility that can accompany obesity. In keeping with this idea, animals with type 1 diabetes have been observed to have gastrointestinal complications[2].

We examined nNOS IR as a marker of inhibitory motor neurons, as well as ChAT and CalR-IR to identify excitatory motor neurons and interneurons, respectively. The results we obtained are consistent with the current literature[4]. For example, our morphological analysis showed that nNOS-IR neurons exhibit Dogiel type I morphologies, while CalR-IR neurons exhibit both Dogiel type I and II morphologies. Qu *et al*[4] in 2008 showed that about 15% of all myenteric neurons are Dogiel type II and are immunoreactive for CalR. Our current findings show that 14% of all the CalR-IR neurons in the myenteric plexus were Dogiel type II in the CG, and that a similar percentage (16%) existed in the OG.

ChAT is the synthesizing enzyme for the excitatory neurotransmitter acetylcholine, a transmitter not found in inhibitory motor neurons[3,4]. In the current work, we

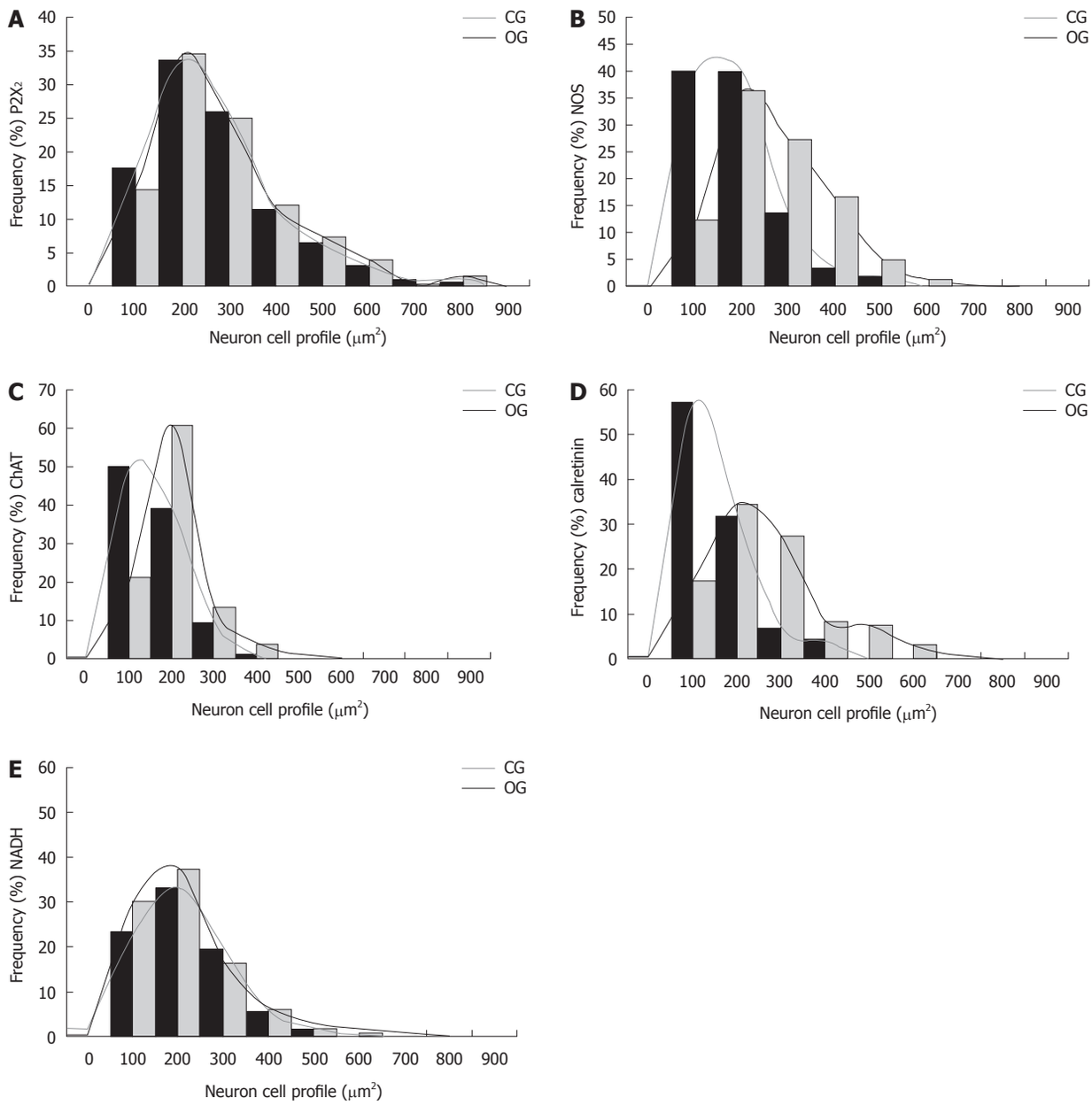


Figure 5 Frequency distribution of neuronal size profiles (μm^2) for neurons immunoreactive for $P2X_2$ receptor (A), nitric oxide synthase (B), choline acetyltransferase (C), calretinin (D), and positive for nicotinamide adenine dinucleotide-diaphorase (E) in the ileum myenteric plexus of control (black bars) and obese (grey bars) groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase; NADH: Nicotinamide adenine dinucleotide; CG: Control group; OG: Obese group.

show that numerous ChAT-IR neurons are present in the myenteric ganglia, as has been described in other species. In addition, we find that all the CalR-IR neurons are also immunoreactive for ChAT, confirming their identity as excitatory cholinergic motor neurons[4].

Changes in neuronal density have been observed in autonomic neurons[24], and various regions of the gastrointestinal tract in rat models of malnutrition and re-feeding[22,23,25] as well as following intestinal ischemia/reperfusion (I/R-i)[18]. In the *ob/ob* group of the current study, the density of nNOS-IR and ChAT-IR neurons was decreased by 42.6% and 53.4% compared to controls; in addition, the total number of nNOS-IR and ChAT-IR neurons was reduced by 35% and 47%, respectively, in the OG compared to controls. No statistically significant difference was found in the density of CalR-IR neurons between the groups. The observed decrease in neuronal density may be explained by the 29% increase in the area of the small intestine that we measured in the obese female mice. However, the estimated overall decrease in the number of neurons in the *ob/ob* intestine implies an actual loss of enteric neurons in these mice. Another enzyme, NADH-diaphorase, has been shown to decrease during malnourishment in neurons of the small and large intestines[22,23]. In the current study, however, we did not observe any alteration in the density of NADH-diaphorase-positive neurons in the OG compared to controls.

In the obese female mouse small intestine, we ob-

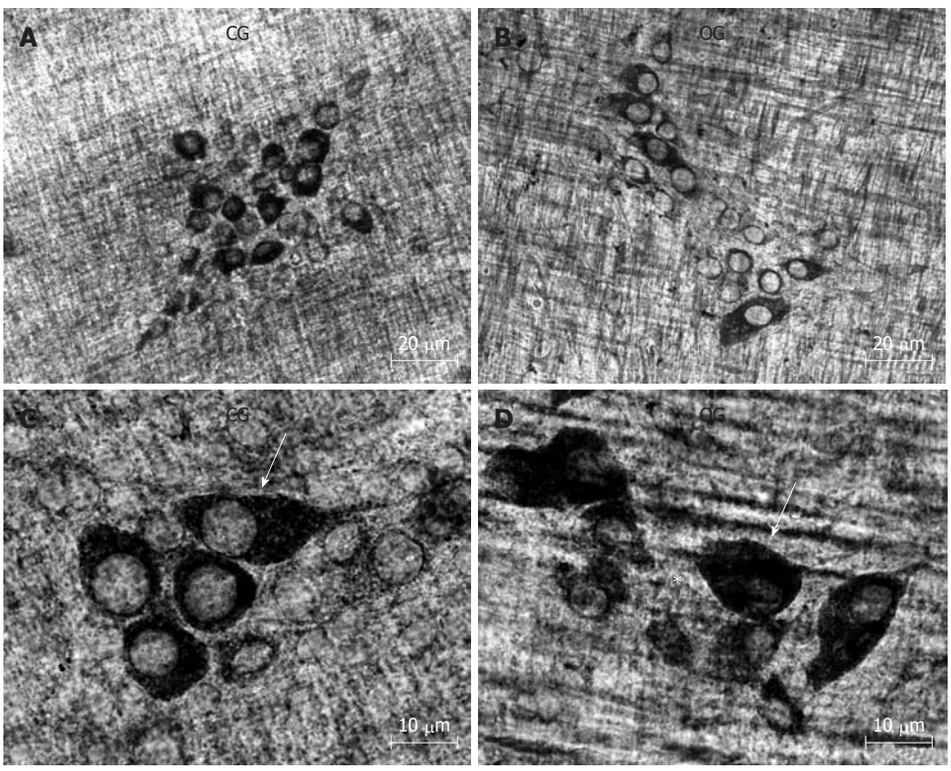


Figure 6 Ileum myenteric neurons labeled for nicotinamide adenine dinucleotide-diaphorase activity in the control group (A, C) and the obese group (C, D), and spaces between the neurons (asterisk) in the obese group group (B, D). Arrows show stained myenteric neurons. CG: **Control group**; **OG**: **Obese group**.

served increases in both the density and estimated number of $P2X_2$R-IR neurons compared to controls. This increase could be due to the presence of $P2X_2$R IR in another type of enteric nervous system cell, one which is not immunoreactive for nNOS, CalR or ChAT; namely, enteric glial cells. The presence of P2X and P2Y receptors has been described in astrocytes and microglia of the central nervous system (CNS)[9] as well as in enteric glial cells[26]. However, the increase of $P2X_2$R IR following undernourishment has been show to occur specifically in enteric neurons[17], making it likely that our observations of increased $P2X_2$R-IR also result from increased neuronal expression.

Changes in the central neuronal expression of $P2X_{1-7}$ purinoceptors frequently occur not only during maturation and neuronal differentiation, but also following various types of acute insults to the CNS. Prolonged stimulation of ATP receptors has been shown to result in changes in the location and density of P2 receptors in the cell membrane[27].

The NADH-diaphorase method is widely used in studies of alterations in the enteric nervous system and other autonomic plexus that relate to undernourishment, re-feeding, and age[22,23,28]. Our current data indicate that the density of NADH-diaphorase positive neurons showed a tendency to decrease in the obese mice compared to controls; however, these differences were not statistically significant.

Previous immunohistochemical and histochemistry studies have shown that under-nutrition[22,23] and I/R-i[18] affect the size profiles of neurons in the gastrointestinal tract. When using NADPH-diaphorase histochemistry to label myenteric neurons, we did not observe differences between the control and OGs, although the range of profile areas using this technique was consistent with that estimated from measurements of nNOS-, ChAT- and CalR-IR. Using an immunohistochemistry technique we were unable to verify exactly which neuronal classes demonstrated alterations in cell size in the obese mouse intestine. We show that cell area was increased for nNOS-, ChAT-, and CalR-IR neurons in the obese mouse, compared to controls. In agreement with these findings, modest (4.4%) increases in the profile area of myenteric neurons have been demonstrated in diabetic rats[29].

Epidemiological studies have suggested that excess body weight may be a major risk factor for colon and breast cancer. In an animal model of colon carcinogenesis, mice fed a high-fat diet exhibited a greater number of colon tumors than lean animals. Their increased abdominal fat was associated with higher concentrations of leptin, insulin, and insulin-like growth factor 1, which possibly mediate tumor growth. These data suggest that the metabolic burden created by excess adiposity accelerates uncontrolled cell growth and survival, thereby increasing the risk of developing breast and colon cancer[30].

Our understanding of the anatomical and molecular consequences of obesity becomes even more complex when we take into account the very complicated nature of the innervation of the gut. We believe that the current

study provides a positive step in our knowledge of the relationship between obesity, diabetes, and gastrointestinal disease. The results of this study suggest that there are significant changes in the intestinal ileum of obese mice, findings that further promote our understanding of obesity and the enteric nervous system.

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COMMENTS

Background

Obesity is a chronic heterogeneous disorder characterized by abnormal or excessive fat accumulation that presents a risk to health. Obesity is associated with increased risk of type 2 diabetes and is often accompanied by complications of the gastrointestinal tract, such as gastroparesis, constipation, diarrhea, and fecal incontinence. In addition, defects in enteric nervous system function have been reported in *ob/ob* mice, including alterations in intestinal motility and in the enteric neurons.

Research frontiers

The myenteric plexus is affected in obesity. The chemical coding of enteric neurons, as well as $P2X_2$ receptor ($P2X_2R$) expression, has not been characterized in the *ob/ob* mice.

Innovations and breakthroughs

The present study showed the effects of obesity in *ob/ob* mice on the morphology of the $P2X_2R$-, nitric oxide synthase-, calretinin- and choline acetyltransferase-immunoreactivity (IR) neurons of the myenteric plexus.

Applications

The present study suggests that obesity may change the chemical coding of the $P2X_2R$-IR neurons in the myenteric plexus, as well as that of inhibitory, cholinergic, and intrinsic primary afferent neurons on the enteric nervous system.

Terminology

The enteric nervous system is composed of the myenteric and submucosal plexuses, which control the motility of the intestine. Adenosine 5'-triphosphate is a well-known neurotransmitter and a ligand of the P2X receptor family, which is made up of seven known receptor subunits PX_{1-7}.

Peer review

This study fulfills its purpose and provides good results both qualitatively and quantitatively.

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ORIGINAL ARTICLE

Emerging roles of the ribonucleotide reductase M2 in colorectal cancer and ultraviolet-induced DNA damage repair

Ai-Guo Lu, Hao Feng, Pu-Xiong-Zhi Wang, Ding-Pei Han, Xue-Hua Chen, Min-Hua Zheng

Ai-Guo Lu, Hao Feng, Pu-Xiong-Zhi Wang, Min-Hua Zheng, Shanghai Minimally Invasive Surgery Center, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China
Ding-Pei Han, Department of Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, China
Xue-Hua Chen, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, China
Author contributions: Lu AG and Feng H contributed equally to this work; Feng H, Lu AG and Zheng MH designed the research; Feng H, Lu AG and Wang PXZ performed the research with Chen XH; Feng H and Han DP analyzed data; Feng H and Lu AG wrote the manuscript; Lu AG and Zheng MH revised and finally approved the article to be published.
Correspondence to: Dr. Hao Feng, Shanghai Minimally Invasive Surgery Center, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China. fengzi.x@163.com
Telephone: +86-21-63846590 Fax: +86-21-63846590
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Abstract

AIM: To investigate the roles of the ribonucleotide reductase M2 (RRM2) subunit in colorectal cancer (CRC) and ultraviolet (UV)-induced DNA damage repair.

METHODS: Immunohistochemical staining of tissue microarray was performed to detect the expression of RRM2. Seven CRC cell lines were cultured and three human colon cancer cell lines, i.e., HCT116, SW480 and SW620, were used. Reverse transcription polymerase chain reaction and Western blotting were performed to determine the mRNA and protein expression levels of RRM2, respectively. Cell proliferation assay, cell cycle analysis were performed. Cell apoptosis was evaluated by double staining with fluorescein isothiocyanate-conjugated Annexin Ⅴ and propidium iodide (PI) using Annexin Ⅴ/PI apoptosis kit. The motility and invasion of CRC cells were assessed by the Transwell chamber assay. Cells were irradiated with a 254 nm UV-C lamp to detect the UV sensitivity after RRM2 depletion.

RESULTS: Immunohistochemical staining revealed elevated RRM2 levels in CRC tissues. RRM2 overexpression was positively correlated with invasion depth ($P < 0.05$), poorly differentiated type ($P = 0.0051$), and tumor node metastasis stage ($P = 0.0015$). The expression of RRM2 in HCT116 cells was downregulated after transfection, and HCT116 cell proliferation was obviously suppressed compared to control groups ($P < 0.05$). In the invasion test, the number of cells that passed through the chambers in the RRM2-siRNA group was 81 ± 3, which was lower than that in the negative control (289 ± 7) and blank control groups (301 ± 7.2). These differences were statistically significant ($P < 0.01$). Our data suggest that RRM2 overexpression may be associated with CRC progression. RRM2 silencing by siRNA may inhibit the hyperplasia and invasiveness of CRC cells, suggesting that RRM2 may play an important role in the infiltration and metastasis of CRC, which is a potential therapeutic strategy in CRC. In addition, RRM2 depletion increased UV sensitivity.

CONCLUSION: These findings suggest that RRM2 may be a facilitating factor in colorectal tumorigenesis and UV-induced DNA damage repair.

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Key words: Ribonucleotide reductase M2; Colorectal cancers; Tissue microarray; Ultraviolet irradiation; Cancinogenesis; Metabolic genes

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Polymorphism, The Catholic University Medical College, 505 Banpo-dong, Socho-gu, Seoul 137-701, South Korea

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INTRODUCTION

Colorectal cancer (CRC) is the leading cause of cancer-related death worldwide. In addition, the incidence and mortality rates of CRC are on the rise[1]. CRC develops through a specific series of events, from the transformation of normal colonic epithelium to an adenomatous intermediate, and then ultimately adenocarcinoma[2]; however, the molecular mechanism underlying CRC metastasis is still not fully understood. With evolving insights into the biology of colorectal neoplasia and advances in assay technology, molecular tests are now being developed as accurate, user friendly, safe, and affordable screening tools[3]. For the accurate diagnosis and adequate treatment of CRC, the identification and understanding of the molecules responsible for cancer progression are critical.

Given that all cancer cells rely on changes in metabolism to support their growth and survival, targeting metabolism has the potential to affect cancers arising from many different tissues. Ribonucleotide reductase (RR) is one of these target metabolic enzymes for cancer therapy. RR transforms RNA building blocks to DNA building blocks by catalyzing the substitution of the 2' OH-group of a ribonucleotide with a hydrogen by a mechanism involving protein radicals[4]. In humans, bacteria, and yeast, RR is a highly regulated enzyme in the deoxyribonucleotide synthesis pathway[5]. Human RR is an enzymatic complex that consists of two nonidentical subunits, M1 and M2. M2 has two alternative subunits; namely, ribonucleotide reductase M2 (RRM2), which is involved in DNA replication (the focus of this paper), and p53R2, which is induced by p53 and is involved in supplying dNTPs for mitochondrial DNA replication and repair[6]. In addition, RRM2 is the limiting factor of RR enzymatic complex catalytic activity, which is specifically expressed in the S phase[7,8]. Therefore, RR plays an important role in the regulation of cell proliferation[9-11]. *In vitro* studies have shown that aggressive tumor proliferation results in overamplification of RR[12,13]. In some types of cancer, elevated RRM2 levels correlate with chemoresistance[14], cellular invasiveness[15], and poor patient outcome[16], which suggests that RRM2 may contribute to malignant progression, and thus be a potential therapeutic target. Recent findings have established that p53R2 suppresses the invasiveness of cancer cells, and its expression is associated with a better survival prognosis for CRC patients[17]; however, the function of RRM2 in CRC is unclear. Here, we demonstrate that RRM2 may play an important role in the development of CRC and may contribute to the response to ultraviolet (UV) irradiation.

MATERIALS AND METHODS

Patients, tissue microarray and histological analysis

This study consisted of a tissue microarray (TMA, Shanghai Outdo Biotech, National Engineering Center for Biochip at Shanghai, China) containing 56 CRC tissues and 56 adjacent normal mucosa, which were laparoscopically resected at Shanghai Minimal Invasive Surgery Centre (Shanghai Jiao Tong University School of Medicine) between 2007 and 2009. In addition, another 78 specimens of CRC tissues were laparoscopic resected between 2011 and 2012. None of the patients received preoperative treatment such as radiation or chemotherapy. All research protocols in the present study were approved by the Institutional Review Board. Staging of the tumors was performed according to the tumor node metastasis classification updated by the American Joint Commission on Cancer and the World Health Organization[18]. Pathological diagnoses were verified by two different pathologists. TMA blocks (5 μmol/L) were sectioned. Preparation of sections from paraffin blocks was performed as previously described[19]. Immunohistochemical and immunocytochemical analysis of RRM2 was performed with an anti-RRM2 antibody [R2 (E-16): SC-10846] (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, United States). Relative RRM2 expression and subcellular location were classified into 5 groups based on the intensity of RRM2 staining and its distribution between the cytoplasm and nucleus.

Cell lines and culture conditions

Seven human CRC cell lines were purchased from American Type Culture Collection (Manassas, VA, United States). The CRC cell lines, HCT116 and HT-29, were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, L-glutamine, and nonessential amino acids. SW1116, SW620, SW480, LoVo and Colo205 cells were maintained in the same medium, except that McCoy's 5A medium was substituted with RPMI 1640 medium (Sigma Aldrich, St Louis, MO, United States). Cells were maintained at 37 ℃ in a 5% CO_2 incubator, and were grown according to standard protocols.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. Reverse transcription was performed with oligo dT primers. Real-time polymerase chain reaction (PCR) was carried out in an Applied Biosystems 7500 System with Power SYBR Green PCR Master Mix (2 ×, Applied Biosystems, Warrington, United Kingdom). Relative levels of gene expression were determined with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control.

RNA interference

Transfection of cells with siRNAs targeting RRM2 was

carried out by mixing the siRNAs with diluted Lipofectamine™ 2000 at a final siRNA concentration of 20 nmol/L. The target sequences for siRNAs were as follows: 5'-AAACCCGAGGAGAGAUAUUTT-3', 5'-GGAGCGAUUUAGCCAAGAATT-3' and 5'-GCACUCUAAUGAAGCAAUATT-3'. siRNAs targeting RRM2 and a negative control siRNA (5'-UUCUCCGAACGUGUCACGUTT-3') were purchased from Genepharma (Shanghai, China). Each experiment was performed in triplicate.

Lentiviral shRNA vector

The following sets of oligonucleotides were used to knockdown RRM2 expression; shRRM2-sense: CACCG GAGCGATTTAGCCAAGAAGTCACCACTTCTTGG CTAAATCGCTCC and shRRM2-antisense: AAAAGG AGCGATTTAGCCAAGAAGTGGTGACTTCTTGG CTAAATCGCTCC. Each set of oligonucleotides were annealed and cloned into pGPH1/Neo (Genepharma, Shanghai, China).

Western blot analysis

An anti-RRM2 antibody (0.2 μg/mL) or goat polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology; 0.2 μg/mL) was used as the primary antibody. Seven human CRC cell lines were resuspended in ice-cold NP-40 lysis buffer, and cell extracts from the colorectal mucosa were prepared by scraping mucosal epithelial cells into ice-cold NP-40 lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EGTA, and 1% NP-40) containing protease inhibitors. The isolated protein was quantified by a commercially available modified Bradford assay (Bio-Rad Laboratories, Hercules, CA, United States).

Cell viability assay

Cells were seeded at 2.5×10^3 cells per well in 96-well plates and cultured for 120 h. Cells were counted using the CASY TT Cell Counter (Roche Diagnostics, Indianapolis, United States). Cell viability was determined by adding 10 μL of Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) to each well at 37 ℃ in a 5% CO_2 incubator. Each experiment was performed three times. 96-well plates were read using an Epoch Microplate Spectrophotometer (BioTek, Winooski, United States) with Gen5™ Microplate Data Analysis Software included.

Invasion assays

Motility and invasion capabilities *in vitro* were measured in a transwell chamber assay (Becton Dickinson Labware, Bedford, MA, United States). HCT116 cells (2.5×10^5) were seeded in McCoy's' 5A medium without FBS, and then plated on each 8.0 μmol/L pore size membrane insert in 24-well plates. McCoys' 5A medium plus 20% FBS was plated in the bottom wells as chemoattractants. After 24 h, cells on the top side of the inserts were removed with a cotton swab. Cells that had migrated to the underside of the inserts were stained with 0.05% crystal violet for 1 h before rinsing with phosphate buffered saline (PBS) for 20 min. Cells on each insert were counted at a 20 × magnification in 10 random fields of view. The invasion assay was done in the same way except that the 8.0-μmol/L pore size membrane insert was coated with Matrigel that was diluted in McCoys' 5A medium (1:8 dilution). The results were expressed as mean ± SD.

Flow cytometric analysis

HCT116 cells after different treatments were rinsed twice with saline PBS. Cells were dissociated, followed by the addition of 5 μL Annexin V-FITC. Cells were then gently vortexed and incubated for 15 min at 4-8 ℃ in the dark. Propidium iodide (PI, 10 μL) was added to the tube for another 5 min at 4-8 ℃ in the dark. The labeled cells were analyzed using the BD FACS Vantage System (Becton, Dickinson and Company, Franklin Lakes, NJ, United States) in accordance with the manufacturer's protocol. Gating was implemented on the basis of the staining profiles of the negative control.

UV irradiation

Cells were irradiated with a 254 nm UV-C lamp (UVP Inc., Upland, CA), and doses were measured with a UVX radiometer (Upland).

Statistical analysis

All continuous values were expressed as mean ± SD and all experiments were repeated three times. The results were subjected to a nonparametric Mann-Whitney *U* test. A paired Student's test was also used to analyze the intragroup differences. All statistical analyses were done using Stat View 5.0 for Windows (SAS Institute Inc., Cary, NC, United States). Fisher's exact test and Cochram-Artimage test were applied to analyze the relationship between RRM2 immunoreactivity and clinicopathological features. Student's *t*-test was also used to test differences in cell viability assays. A P value less than 0.05 was considered statistically significant.

RESULTS

Overexpression of RRM2 in CRC tissues and clinicopathologic significance of RRM2 expression in CRC patients

To examine RRM2 protein expression in normal mucosa and CRC tissues, we performed hRRM2 immunohistochemistry on the TMA. The normal human colon exhibits occasional $RRM2^+$ cells located at the base of the crypt (six cases). In comparison, 38 of 56 tumor samples exhibited a substantial increase in overall RRM2 expression and in the number of $RRM2^+$ cells. In the majority of these tumors, RRM2 was present in the cytoplasm (Figure 1). We set the cutoff point of RRM2 overexpression as 10%, because this is obviously higher than the positivity in normal colorectal mucosa, and because it was easy to determine positive or negative expression with this simple cutoff value. Among the 56 CRC tissues included in the TMA, RRM2 overexpression (⩾ 10% cancer cells stained) was observed in 38 cases (67.9%). The

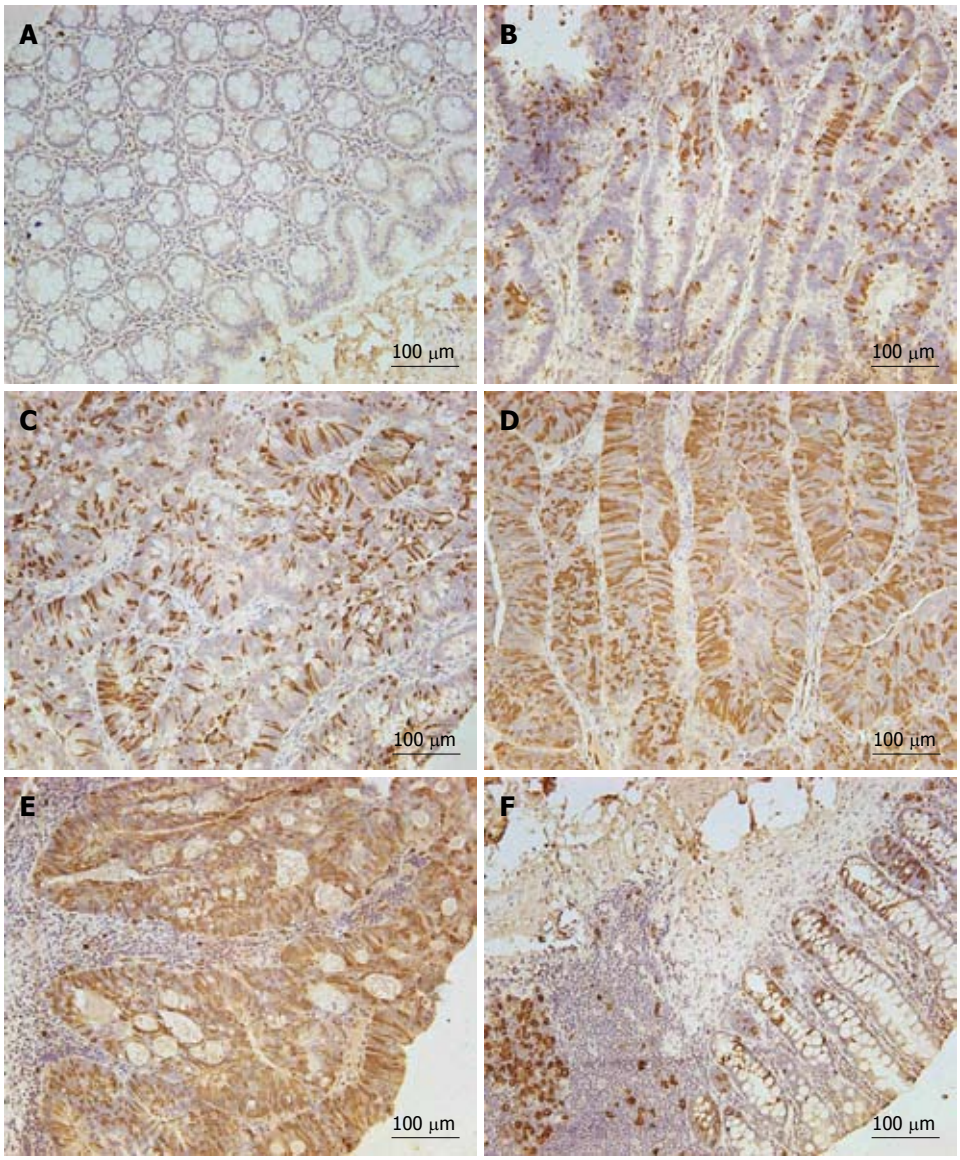


Figure 1 Immunohistochemical analysis of ribonucleotide reductase M2 expression in surgical specimens of tumor tissues or adjacent normal mucosa from patients with colon cancer. A: Normal colorectal mucosa without ribonucleotide reductase M2 (RRM2) expression. This case was considered RRM2-negative; B: Lower RRM2 expression in colorectal cancer (CRC); C-E: High RRM2 expression in CRC; F: Lymph node metastasis of CRC with RRM2 overexpression (original magnification × 20).

relationship between RRM2 expression and clinicopathologic characteristics in CRC patients is summarized in Table 1. RRM2 overexpression was significantly positively correlated with invasion depth ($P = 0.0015$) and differentiation ($P = 0.0051$), although the frequency of RRM2 overexpression was lower in T4 tumors (20%) compared to T3 tumors (75%). However, no significant differences were observed with respect to gender, age, tumor size, or lymph node metastasis. To further elucidate the clinicopathologic significance of RRM2 overexpression in CRC, especially the correlation with invasion depth and poor differentiation, we examined RRM2 expression in another 78 CRC tissues. The results are summarized in Table 2 and RRM2 immunostaining was categorized further as score 0-3[20]. These data are in accordance with previous studies showing correlation between RRM2 overexpression, invasion depth ($P < 0.0001$) and differentiation ($P < 0.0001$).

Suppression of RRM2 inhibits CRC cell growth and invasion

We next sought to determine whether RRM2 overexpression is functionally important for the *in vitro* proliferation of colonic cancer cell lines. RRM2 had the highest expression levels in HCT116 cells (Figure 2A and B). Then, HCT116 cells were transfected with three different siRNA sequences or a scrambled siRNA control. Quantitative PCR analysis of the HCT116 cell line demonstrated that the expression of RRM2 mRNA was reduced by three siRNA oligos segments to varying degrees (Figure 2C). We used the most efficient one to down-regulate RRM2 and evaluated cell growth; efficient knockdown of RRM2 protein levels after transfection was observed (Fig-

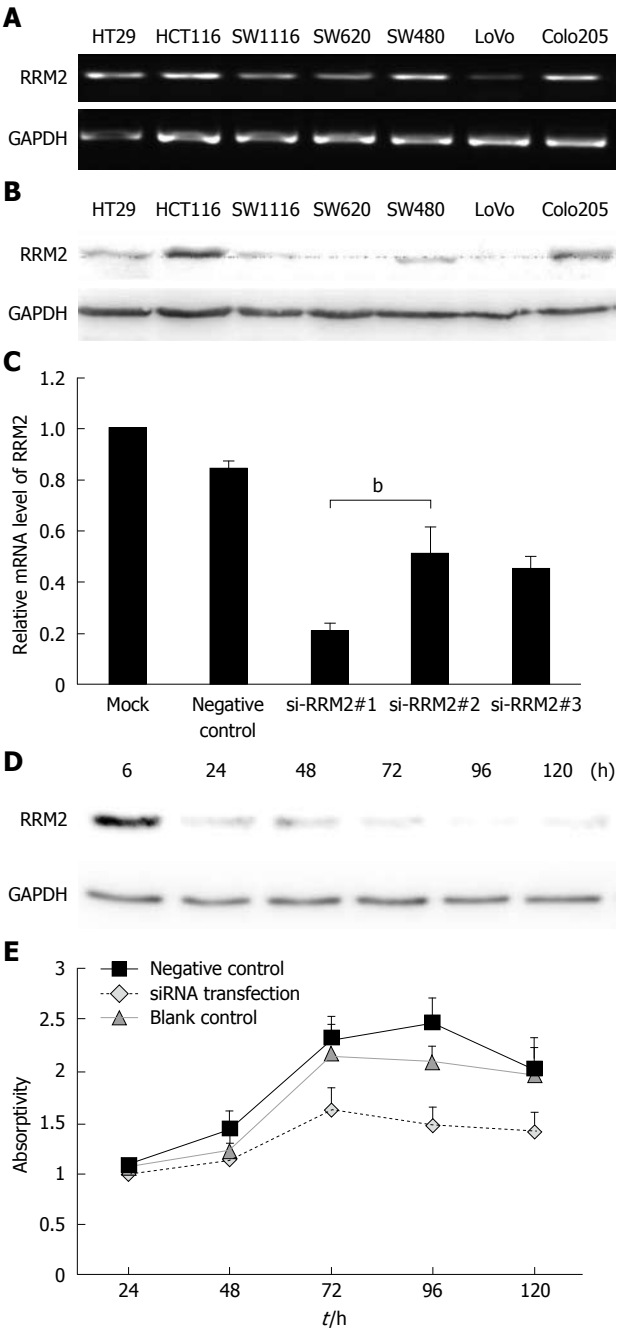


Figure 2 Expression of ribonucleotide reductase M2 in colorectal carcinoma cell lines and effect of ribonucleotide reductase M2 depletion on cell proliferation. A: Ribonucleotide reductase M2 (RRM2) expression in seven colorectal cancer (CRC) cell lines (PCR analysis); B: Expression of RRM2 protein in seven CRC cell lines (Western blotting); C: 48 h after siRNA transfection, relative mRNA level of HCT116 was determined (mock: lipofectamine only, negative control: lipofectamine + control siRNA), $^{b}P < 0.01$ si-RRM2#1 *vs* si-RRM2#2; D: HCT116 cells were transfected with si-RRM2#1 and cultured for 120 h. Cells were lysed and RRM2 protein levels were detected by Western blotting. Glyceraldehyde 3-phosphate dehydrogenase expression was used to control equal protein loading; E: At 24-120 h after siRNA transfection, the viability of HCT116 cells was determined by the CCK-8 assay. Analysis of cell growth in the presence or absence of RRM2 siRNAs revealed that blocking RRM2 protein synthesis significantly inhibited HCT116 cell growth. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

ure 2D). Analysis of cell growth in the presence or absence of the RRM2 siRNAs revealed that blocking RRM2 protein synthesis significantly inhibited CRC cell growth (Figure 2E). We used Annexin V/PI staining to identify the dead cells and then stratified them into categories of necrosis, early apoptosis, and both later apoptosis and necrosis. In each flow cytometry graph, later apoptotic or necrotic cells are shown in the upper-right quadrant. The total death rate 48 h after siRNA treatment showed a 2-3 fold increase. Flow cytometric analysis showed evidence of apoptosis when RRM2 functions were abrogated (Figure 3A and B). In the invasion assay, the number of cells that crossed through the chambers in the RRM2-siRNA group was 81 ± 3, which was lower than that in the negative control (289 ± 7) and empty control groups (301 ± 7.2). These differences were statistically significant ($P < 0.01$) (Figure 3C and D).

Table 1 Relationship between ribonucleotide reductase M2 gene expression in tumor and pathological parameters in patients *n* (%)

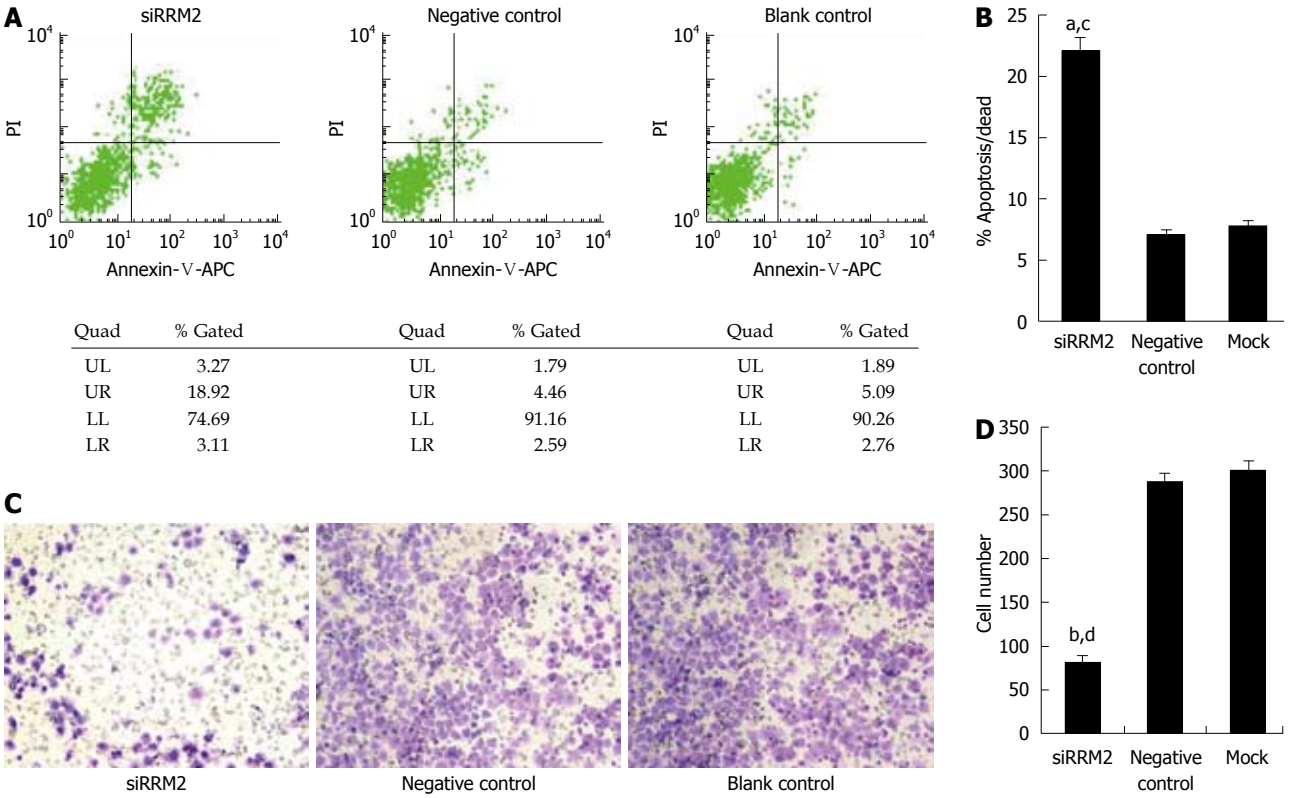
Clinical or pathologic feature	Total (*n*)	RRM2^{+}	*P* value
All cases	56	38 (67.9)	
Gender			0.0908
Male	33	20 (60.6)	
Female	23	18 (78.3)	
Age (yr)			0.2094
≤ 64	29	19 (65.5)	
≥ 65	27	19 (70.4)	
Tumor stage (invasion depth)			0.0015
Tis/T1	3	0	
T2	16	6 (37.5)	
T3	32	24 (75.0)	
T4	5	1 (20.0)	
Tumor size (cm^2, average = 17.3)			0.0677
≤ 17	31	20	
> 17	25	11	
Lymph node metastasis (N)			0.2281
Absent	25	14 (56.0)	
1-3	21	16 (76.2)	
≥ 4	10	8 (80.0)	
Classification (differentiation)			0.0051
High-grade	5	1 (20.0)	
Mid-grade	45	35 (77.8)	
Low-grade	6	2 (33.3)	

RRM2: Ribonucleotide reductase M2.

Table 2 Relationship between ribonucleotide reductase M2 overexpression with invasion depth and poor differentiation

Pathologic feature	Total (*n*)	RRM2^{-}		RRM2^{+}		DF	*P* value
		Score 0	Score 1	Score 2	Score 3		
Tumor stage (invasion depth)						1	< 0.0001
Tis/T1	17	12	4	1	0		
T2	26	1	6	16	3		
T3	21	0	2	8	11		
T4	14	0	2	5	7		
Classification (differentiation)						1	< 0.0001
High	20	9	9	2	0		
Mid	39	4	3	20	12		
Low	19	0	2	8	9		

RRM2: Ribonucleotide reductase M2; DF: Degrees of freedom.



Quad	% Gated	Quad	% Gated	Quad	% Gated
UL	3.27	UL	1.79	UL	1.89
UR	18.92	UR	4.46	UR	5.09
LL	74.69	LL	91.16	LL	90.26
LR	3.11	LR	2.59	LR	2.76

Figure 3 Effect of ribonucleotide reductase M2 on apoptosis and invasive capacity. A: Increased apoptosis following ribonucleotide reductase M2 (RRM2) mRNA inhibition by RNAi. Percentages of cells in the bottom left quadrant are live while cells to the right of the vertical line are apoptotic or dead; B: Quantifying bar graphs showing a significant increase in the percentage of apoptotic/dead cells following RRM2 mRNA knockdown with siRNA, $^{a}P < 0.05$ *vs* negative control; $^{c}P < 0.05$ *vs* blank control; C: Effect of RRM2 on invasive capacity of colorectal cancer cells. Invasion through the Matrigel-coated collagen insert after the indicated treatments is shown; D: Quantifying bar graphs showing a significant decrease in cell number following RRM2 mRNA knockdown with siRNA, $^{b}P < 0.01$ *vs* negative control; $^{d}P < 0.01$ *vs* blank control. Quad: Quadrant; UL: Upper left; UR: Upper right; LL: Lower left; LR: Lower right. PI: Propidium iodide.

Inhibition of hRRM2 by expression of antisense hRRM2 results in increased UV sensitivity of CRC cells

Four human CRC cell lines with low RRM2 expression, namely, SW1116 (p53 mutant-type), SW620 (mutated p53), HT29 (mutated p53), SW480 (p53 mutant-type), and LoVo (p53 wild-type), all proved to be more sensitive to UV irradiation in colony formation assays than HCT116 cells (p53 wild-type). HCT116 cells transfected with shRRM2 revealed significant RRM2 depletion, and its colony-forming ability under UV radiation was suppressed. At a dosage of 10 J/m^2, the colony-forming ability of the HCT116 cells transfected with RRM2 antisense was inhibited by 93.13%, whereas wild-type HCT116 cells maintained 51.28% viability, and SW620 cells (RRM2-/-) maintained 32.40% viability (Figure 4A and B). In addition, the cell cycle distribution of the CRC cells did not significantly change at the various time points in the UV (–) panel. After a sublethal dose of UV radiation, an increased number of cells in the S-phase could be seen at 24-36 h (Figure 4C). Cellular morphology was observed by light microscopy. HCT116 cells with high levels of RRM2 expression, and SW480 cells, which do not express RRM2, were cultured under normal conditions or exposed to UV radiation for 48 h. Under these conditions, SW480 cells showed UV hypersensitivity, whereas the UV sensitivity of HCT116 cells with suppressed RRM2 levels increased and cells went through necrosis. Thus, endogenous RRM2 functions to suppress hypersensitivity to UV radiation. Figure 5A gives a visual depiction of the morphological changes that occurred.

DISCUSSION

The importance of altered metabolism in the context of tumorigenesis has received renewed attention as metabolic changes entwined with the molecular hallmarks of cancer have been elucidated. Previous studies have shown that alterations in RR levels may significantly influence the biological properties of cells, including tumor promotion and tumor progression, suggesting that RR may be implicated in tumorigenesis. Thus, we were interested in determining the effect of RRM2 on tumor cell growth. The discovery of p53R2, an analog of RRM2 in mammalian cells, provided an interesting link between RR and cancer because it is a downstream target for the p53 tumor suppressor. Previous studies have shown that p53R2 is negatively related to tumor invasion and lymph node involvement. In addition, investigations have revealed that p53R2 correlates with a better survival of CRC with advanced grade Ⅲ and grade Ⅳ tumors, rather than earlier grade Ⅰ and grade Ⅱ tumors[18]. The genes for p53R2 and RRM2 are located on separate chromosomes.

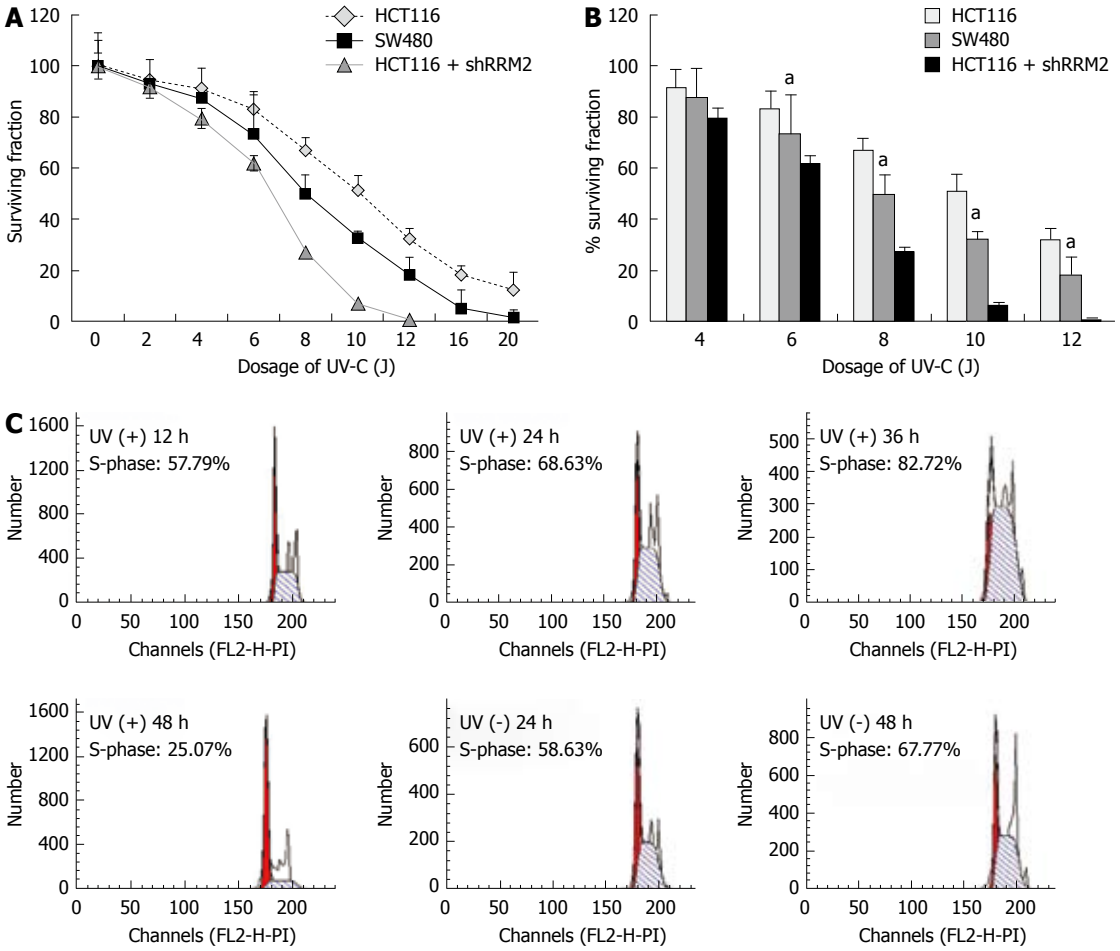


Figure 4 Inhibition of ribonucleotide reductase M2 by expression of antisense hRRM2 results in increased ultraviolet sensitivity of colorectal cancer cells. A: Ribonucleotide reductase M2 (RRM2)-/- tumors and RRM2-depleted cells are hypersensitive to ultraviolet (UV)-C. SW620 cells, HCT116 cells (WT), and HCT116 cells (RRM2-/-) were irradiated with increasing doses of UV-C and assayed for colony formation; B: Bar graph showing the quantification of the surviving fraction following 4-12 J irradiation. HCT116 cells transfected with shRRM2 revealed significant RRM2 depletion, and its colony-forming ability under UV radiation was suppressed, $^{a}P < 0.05$ *vs* HCT116 group; C: Effects of colorectal cancer cell cycle irradiated with UV-C. After UV irradiation, an S-phase increase could be seen, starting from 12 h with a continued significant increase at 24 and 36 h.

p53R2 is 80%-90% identical to RRM2, but lacks its 33 amino-terminal residues, including a KEN box required for degradation during mitosis. p53R2 can substitute for RRM2 and form a highly active RR, which is believed to be active in DNA repair after DNA damage[4]. However, the current knowledge about RRM2 function in human CRCs is still preliminary[20,21]. Therefore, determining if RRM2 is essential for CRC progression may provide promising therapeutic opportunities.

Our findings showed that RRM2 expression is substantially upregulated in CRC using microarray analysis and RT-PCR. Moreover, RRM2 overexpression was significantly associated with invasion depth and differentiation, and clinical tissue specimens also showed that the expression levels of RRM2 may be associated with tumor stage (Figure 5B). These data suggest that RRM2 overexpression could be associated with CRC progression. Several previous reports demonstrated the frequent overexpression of the RRM2 protein and its possible role in other cancers[20-23]. Recent observations showed that top 400 gene lists is produced by microarrays for each of the 13 cancer types and RRM2 appeared in nine lists of significantly altered genes in cancers[23], the results point to the value of RRM2 in normal to cancer transformation. RRM2 is upregulated in esophageal/gastric cancers and melanomas compared to pancreatic cancer[24]. Other groups have also suggested that RRM2 may be a novel diagnostic marker and potential therapeutic target in bladder and gastric cancers. Furthermore, the studies suggested that RRM2 overexpression is associated with the male sex, the presence of muscularis propria invasion, the presence of Epstein-Barr virus, and expression of survivin, but not with age, histology, tumor size, lymph node metastasis, or expression of the phosphatase and tensin homolog, the phosphorylated signal transducer, and p53. Suppression of RRM2 synthesis inhibited the growth of gastric cancer cell lines *in vitro*[20]. In addition, RRM2 is one of the putative tumor markers that is strongly expressed in the very early stages of premalignancy and preneoplasia of breast carcinomas[25], and hepatocellular carcinoma cells are highly dependent on these genes for proliferation[26]. Analyses also revealed that hRRM2 positively correlates with poor tumor differentiation of non-small cell lung cancer[27]. It may be that

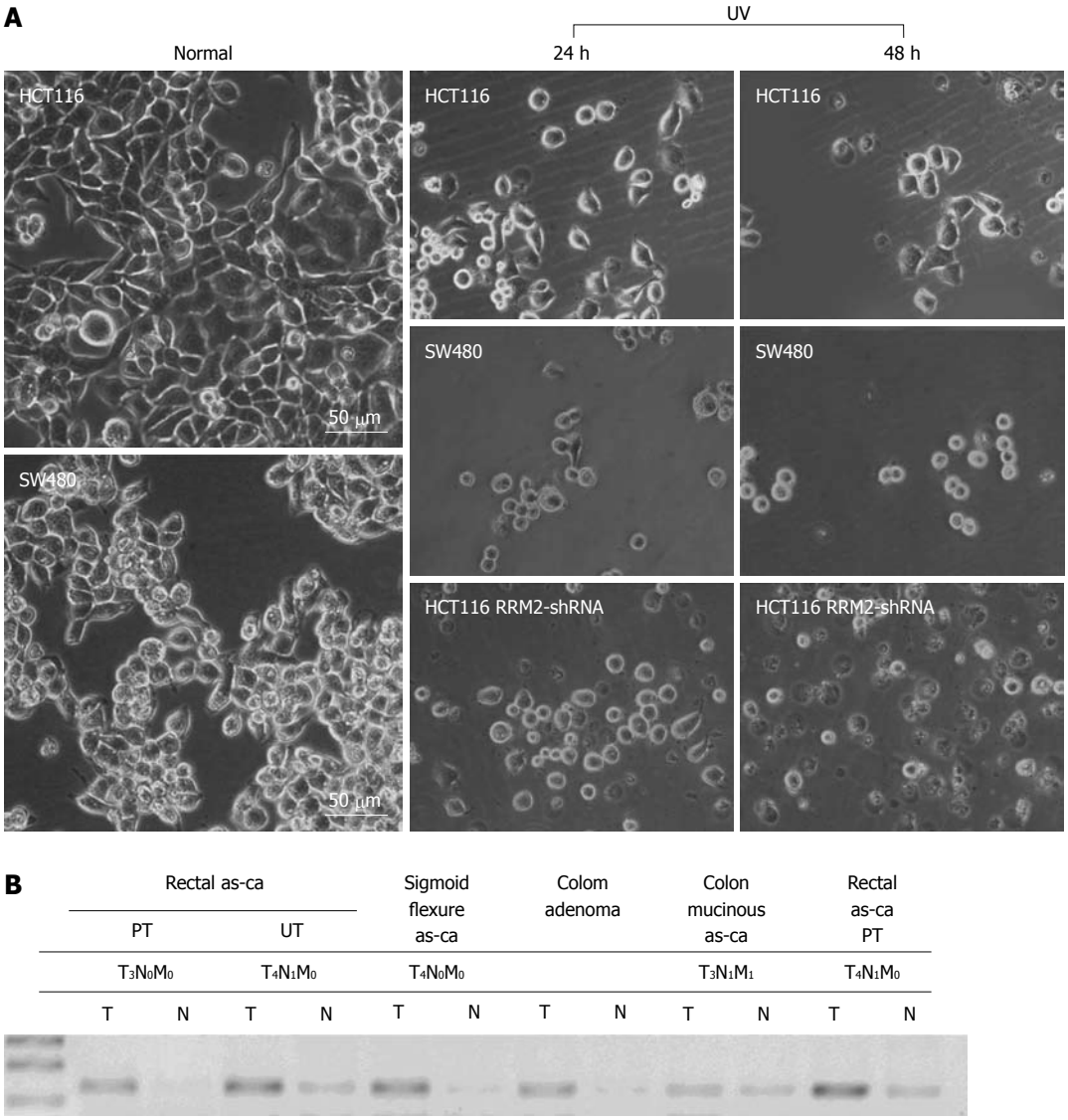


Figure 5 Light-microscopic photograph of cellular morphology. A: After 48 h of culture in normal conditions or exposed to ultraviolet (UV), HCT116 cells [high expression level of ribonucleotide reductase M2 (RRM2)] and SW480 cells (low expression level of RRM2) were photographed (20 × magnification). With UV-C irradiating, SW480 cells formed spheroids and HCT116 cells still adhered to each other and attached; B: Expression of RRM2 mRNA in clinical tissue specimens. Polymerase chain reaction analyses of 25 colorectal cancer patients (we show 6 patients) showed the expression levels of RRM2. PT: Protrude type; UT: Ulcerative type; as-ca: Adenocarcinoma.

the altered expression of metabolic enzymes or changes in the regulation of metabolic pathways also occur downstream of many oncogenes and tumor suppressor genes, and cancers with specific genetic lesions are dependent upon some of these metabolic changes. As was previously shown, RRM2 is upregulated in several cancers, and abrogating its expression reduces the proliferation of cancer cells, and triggered the development of RRM2-targeted siRNA-containing nanoparticles.

We also demonstrated that siRNA-mediated suppression of RRM2 synthesis inhibited the growth of the CRC cell lines investigated. In addition, abrogating its expression induced cell apoptosis. Therefore, it is likely that RRM2 overexpression is essential for the survival of CRC cells. Our finding is consistent with recent observations demonstrating that cells with altered RRM2 expression exhibit significantly reduced subcutaneous tumor latency and increased tumor growth rates in syngeneic mice, and show markedly elevated metastatic potential in lung metastasis assays[28]. Their studies demonstrate that RRM2 can participate in other critical cellular functions in addition to ribonucleotide reduction. For example, RRM2 overexpression may increase pancreatic cancer cell invasion in an NF-κB-dependent manner, and RNA interference mediated silencing of RRM2 expression attenuated cell invasion and matrix metallopeptidase 9 activity[16]. Another laboratory also reported that RRM2 overexpression plays an important role in tumor invasiveness[29,30]. Our results are consistent with these previous reports, and suggest that targeting RRM2 has the potential for the treatment of CRC.

RRM2 is known to be involved in chemoresistance. The suppression of RRM2 can sensitize colon cancer cells to chemotherapeutic agents and significantly inhibit

the proliferation of melanoma cell lines with different oncogenic mutations with synergistic enhancement in combination with temozolomide[31]. The relationship between RRM2 expression and chemotherapeutic effects in clinical settings has also been investigated. For instance, GTI-2040, an antisense oligonucleotide targeting RRM2, has been tested for the treatment of advanced metastatic solid tumors in clinical settings[32]. Radiation is also a key component of carcinoma therapy. Unfortunately, it results in significant side effect in some patients. Thus, agents that radiosensitize CRC cells would be very useful. Here we show that RRM2 depletion decreased the surviving fraction of tumor cells in response to radiation and increased the sensitizer enhancement ratios. Recent observations suggest that targeting polo-like kinase 1 (PLK1) with small molecule inhibitors, in combination with radiation therapy, is a novel strategy in the treatment of some cancers[33]. In addition, it has been hypothesized that there are some links between RRM2 and PLK1, because PLK1 was one of the most drastically downregulated genes following RRM2 silencing[34]. Our data suggest RRM2 depletion increased UV sensitivity and make an argument for further exploring the role of RRM2 inhibition in CRC. It will be important to elucidate in detail the specific mechanisms by which RRM2 mediates tumor cell radiosensitivity.

In conclusion, we performed both functional and correlative studies on RRM2 in CRCs and provide strong evidence that RRM2 overexpression may be associated with CRC progression; thus suppressing its function may be a potential therapeutic strategy in CRC.

COMMENTS

Background

Previous studies have shown that alterations in ribonucleotide reductase (RR) levels may significantly influence the biological properties of cells, including tumor promotion and tumor progression, suggesting that RR may be implicated in tumorigenesis. Thus, the authors were interested in determining the effect of ribonucleotide reductase M2 (RRM2) on colorectal cancer (CRC) cells.

Research frontiers

Recently, metabolic genes have received increasing and specific attention due to their potential role in carcinogenesis. Overexpression of RRM2 has been revealed in breast cancer, renal cell carcinoma, prostate cancer and gastric cancer to elevated cellular proliferation and invasiveness.

Innovations and breakthroughs

Few studies have described the correlation between RRM2 and the development of CRC. And the possible mechanism by which RRM2 mediates CRC progression is unclear. It is well established that in some types of cancer, elevated RRM2 levels correlate with chemoresistance, but whether it may contribute to the response to ultraviolet (UV) irradiation is unclear. The results of this study suggest that RRM2 may be a facilitating factor in colorectal tumorigenesis and UV-induced DNA damage repair.

Applications

The data suggests that RRM2 subunit overexpression is associated with the CRC progression. RRM2 silencing may inhibit the hyperplasia and invasiveness of CRC cells. RRM2 may play an important role in the infiltration and metastasis of CRC and that suppression of its function could be a potential therapeutic strategy in CRC.

Terminology

RR is an essential enzyme required for the *de novo* conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, and it plays an important role in DNA synthesis and repair. RR consists of two subunits, RRM1 and RRM2/p53R2.

Peer review

This is an interesting study investigating the importance of the hRRM2 in CRC and UV-induced DNA damage repair.

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BRIEF ARTICLE

Perforated midgut diverticulitis: Revisited

Milan Spasojevic, Jens Marius Naesgaard, Dejan Ignjatovic

Milan Spasojevic, Jens Marius Naesgaard, Dejan Ignjatovic, Department of Gastrointestinal Surgery, Vestfold Hospital, 3103 Tonsberg, Norway
Dejan Ignjatovic, Department of Digestive Surgery, Akershus University Hospital, University of Oslo, 1478 Lorenskog, Norway
Author contributions: Spasojevic M, Naesgaard JM and Ignjatovic D made substantial contributions to conception and design, drafted the article, and revised it critically for important intellectual content; Spasojevic M and Ignjatovic D performed the acquisition, analysis and interpretation of the data; and all authors approved the version to be published.
Correspondence to: Dejan Ignjatovic, MD, PhD, Department of Digestive Surgery, Akershus University Hospital, University of Oslo, 1478 Lorenskog, Norway. dejan.ignjatovic@ahus.no
Telephone: +47-6-7969895 Fax: +47-6-7969040
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Abstract

AIM: To study and provide data on the evolution of medical procedures and outcomes of patients suffering from perforated midgut diverticulitis.

METHODS: Three data sources were used: the Medline and Google search engines were searched for case reports on one or more patients treated for perforated midgut diverticulitis (Meckel's diverticulitis excluded) that were published after 1995. The inclusion criterion was sufficient individual patient data in the article. Both indexed and non-indexed journals were used. Patients treated for perforated midgut diverticulitis at Vestfold Hospital were included in this group. Data on symptoms, laboratory and radiology results, treatment modalities, surgical access, procedures, complications and outcomes were collected. The Norwegian patient registry was searched to find patients operated upon for midgut diverticulitis from 1999 to 2007. The data collected were age, sex, mode of access, surgical procedure performed and number of patients per year. Historical controls were retrieved from an article published in 1995 containing pertinent individual patient data. Statistical analysis was done with SPSS software.

RESULTS: Group Ⅰ: 106 patients (48 men) were found. Mean age was 72.2 ± 13.1 years (mean ± SD). Age or sex had no impact on outcomes (P = 0.057 and P = 0.771, respectively). Preoperative assessment was plain radiography in 53.3% or computed tomography (CT) in 76.1%. Correct diagnosis was made in 77.1% with CT, 5.6% without (P = 0.001). Duration of symptoms before hospitalization was 3.6 d (range: 1-35 d), but longer duration was not associated with poor outcome (P = 0.748). Eighty-six point eight percent of patients underwent surgery, 92.4% of these through open access where 90.1% had bowel resection. Complications occurred in 19.2% of patients and 16.3% underwent reoperation. Distance from perforation to Treitz ligament was 41.7 ± 28.1 cm. At surgery, no peritonitis was found in 29.7% of patients, local peritonitis in 47.5%, and diffuse peritonitis in 22.8%. Peritonitis grade correlated with the reoperation rate (r = 0.43). Conservatively treated patients had similar hospital length of stay as operated patients (10.6 ± 8.3 d *vs* 10.7 ± 7.9 d, respectively). Age correlated with hospital stay (r = 0.46). No difference in outcomes for operated or nonoperated patients was found (P = 0.814). Group Ⅱ: 113 patients (57 men). Mean age 67.6 ± 16.4 years (range: 21-96 years). Mean age for men was 61.3 ± 16.2 years, and 74.7 ± 12.5 years for women (P = 0.001). Number of procedures per year was 11.2 ± 0.9, and bowel resection was performed in 82.3% of patients. Group Ⅲ: 47 patients (21 men). Patient age was 65.4 ± 14.4 years. Mean age for men was 61.5 ± 17.3 years and 65.3 ± 14.4 years for women. Duration of symptoms before hospitalization was 6.9 d (range: 1-180 d). No patients had a preoperative diagnosis, 97.9% of patients underwent surgery, and 78.3% had multiple diverticula. Bowel resection was performed in 67.4% of patients, and suture closure in 32.6%. Mortality was 23.4%. There was no difference in length of history or its impact on survival between Groups Ⅰ and Ⅲ (P = 0.241 and P = 0.198, respectively). Resection was more often performed in Group Ⅰ (P = 0.01). Mortality was higher in Group Ⅲ (P = 0.002).

CONCLUSION: In cases with contained perforation, conservative treatment gives satisfactory results, laparosco-

py with lavage and drainage can be attempted and continued with a conservative course.

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Key words: Intestinal; Small bowel; Jejunum; Ileum; Perforation; Diverticulitis; Conservative treatment

Peer reviewer: Naoki Ishii, MD, Department of Gastroenterology, St. Luke's International Hospital, 9-1 Akashi-cho, Chuo-ku, Tokyo 104-8560, Japan

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INTRODUCTION

Perforated midgut diverticulitis is an uncommon, acquired condition that is only sporadically observed and treated in surgical departments[1]. This could be the reason for the confusion that still exists when clinical manifestations, diagnosis and treatment options are concerned. The evidence at hand does not seem to be satisfactory to provide the answers needed. Furthermore, there are no data on the use of minimally invasive procedures in the treatment of perforated midgut diverticulitis. A previously published review by Chendrasekhar *et al*[2] in 1995 provided individual patient data for all case reports previously published. This article was chosen to be the landmark for this study because of its extensive data, firm grasp of the problem up to 1995, prior to important developments in diagnostics and treatment.

The aim of this study is to provide new data on the evolution of medical procedures and outcomes in patients with perforated midgut diverticulitis based on individual patient data derived from published case reports, original data and the Norwegian patient registry.

MATERIALS AND METHODS

The data obtained in this study came from three different sources: (Group Ⅰ) review of published case reports in the literature after 1995 combined with original data; (Group Ⅱ) data from the Norwegian patient registry; and (Group Ⅲ) case reports from the literature prior to 1995 as historical controls[2].

Case reports and original data (Group Ⅰ)

Search strategy: Two independent researchers with a medical degree performed the search. The Medline database (http://www.ncbi.nlm.nih.gov/PubMed/) was searched for articles published after 1995. An effort to incorporate all available articles not included in Medline was made through the use of the Google search engine (http://www.google.com/). The following key search strategy was used: ("perforated" or "perforation") and [("jejunum" or "jejunal") or ("intestine, small") or ("intestine" and "small") or ("small intestine") or ("small" and "bowel") or ("small bowel")] and ("diverticulum" or "diverticulitis") for medline databases; and ("perforated" or "perforation") and [("jejunum" or "jejunal") or ("intestine" and "small") or ("small intestine" or "intestine") and ("bowel" or "small bowel")] and ("diverticulum" or "diverticulitis") not sigmoid for Google. References did not have to have abstracts. There were no restrictions by language or publication type. English, French, Spanish, Portuguese, Italian, German and the Scandinavian and Slavic languages were translated by the authors, while help was acquired for translations of Turkish and Korean. All references matching the search were identified and screened for retrieval. Duplicate references were removed by a manual search. The retrieved references were scanned by the authors independently.

Selection: The inclusion criterion was original articles or case reports with any study design including at least one patient undergoing any kind of treatment for perforated midgut diverticulitis (Meckel's diverticulitis excluded), containing pertinent individual patient data. Perforated midgut diverticulitis was defined either as histopathologically confirmed in the surgical specimen (operated patients) or where clear radiological evidence was provided in conservatively treated patients. This evidence was usually computed tomography (CT)-based (localized inflammation or thickened wall of the midgut with/without air bubbles, pseudotumor or abscess in the midgut mesentery), or confirmed with a small bowel series, and in some cases, through radiography with contrast medium after abscess drainage. The population selected were 18 years or older.

Data abstraction: Individual data for each patient included the following: age; sex; surgical procedure (if operated); access (laparoscopic/open/combined); number of diverticula (single/multiple); distance from the ligament of Treitz; diverticula in other segments of the gut (jejunum/ileum/colon); length of symptoms prior to hospitalization; fever; peripheral blood leucocytes and C-reactive protein (CRP) at admission; method of diagnosis; hospital stay; complications (general and surgical); reoperation; and outcome. Details of the intervention included any type of treatment (conservative or surgical). Surgical procedure was classified as resection, suture closure or exploration, laparoscopic or open. Other complications of midgut diverticulitis (bleeding, bacterial overgrowth) were not studied.

Vestfold hospital patient data: Patients treated at Vestfold hospital for perforated midgut diverticulitis from March 2008 to December 2010 were included in this series. All patients had an abdominal CT scan at hospitalization. Indication for surgery was made through the evaluation of abdominal tenderness, rebound tenderness, bowel sounds, blood work-up and CT scan. Antibiotics

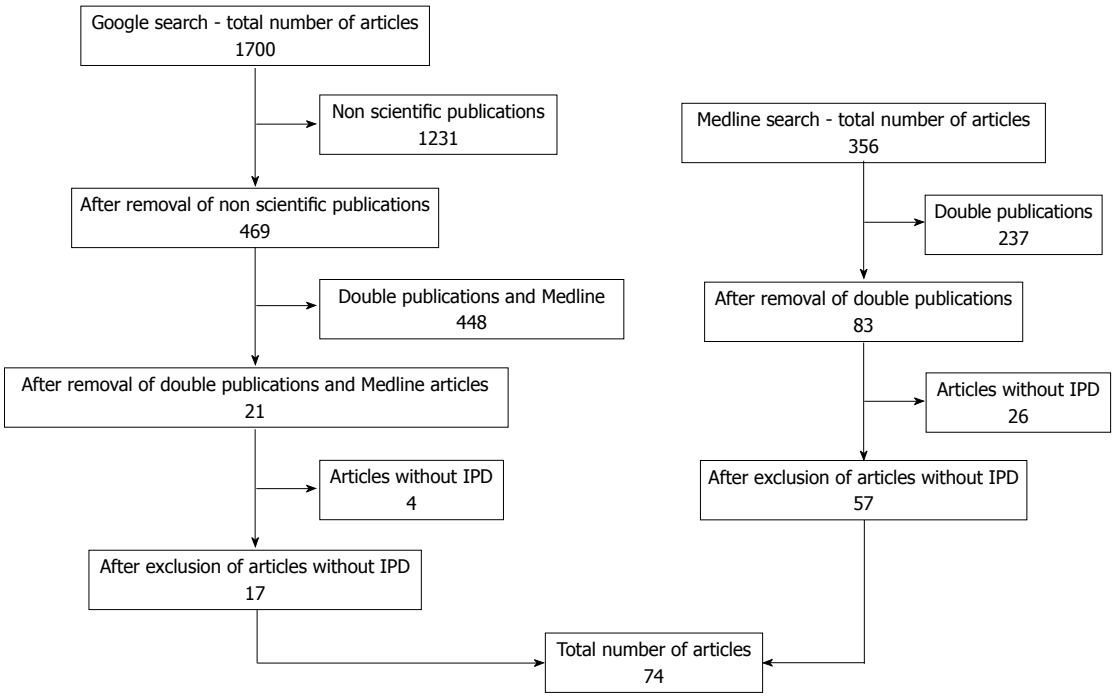


Figure 1 Schematic of the search strategy. IPD: individual patient data.

were administered. Patients who were operated upon had laparoscopy that confirmed the diagnosis, and thereafter a precisely placed minilaparotomy 4-5 cm in length, through which a limited small bowel resection and end-to-end hand-sewn anastomosis was performed. Further investigations (gastroscopy, colonoscopy and small bowel series) were done several weeks after the patient was discharged from the hospital.

Norwegian patient registry (Group Ⅱ)

A comprehensive search of the Norwegian patient registry was performed. The time limit was from 1999 (introduction of the ICD-10 coding system in Norway) to 2007. The search criteria: K57.0 (midgut diverticulitis with perforation or abscess), K57.4 (small and large bowel diverticulitis with perforation or abscess), K57.8 (bowel diverticulitis, unspecified with perforation or abscess), Q43.0 (Meckel's diverticulum) excluded, crossed with JFB00 (small bowel resection), JFB01 (laparoscopic small bowel resection), JFA70 (enterorrhaphy), JFA71 (laparoscopic enterorrhaphy). Patients under the age of 18 years were excluded. The data requested were age, sex, mode of access, surgical procedure performed, and number of patients per year.

Historical controls (Group Ⅲ)

The data on historical controls was retrieved from Chendrasekhar *et al*[2]. Patients under the age of 18 years, and patients that did not have data on age were deleted and the remaining data were copied into the statistical software and used for further analysis.

Statistical analysis

Analysis was done using SPSS statistical software (Chicago, IL, United States). Distribution of data was checked with the Shapiro-Wilks test. Nonparametric values were compared using the χ^2 test and the Mann-Whitney test, while parametric values were compared using Student's *t* test. Correlations were tested with Spearman's test. The *P* values given were two sided, $P < 0.05$ was considered as the limit of significance.

RESULTS

Case reports and patients treated in Vestfold Hospital (Group Ⅰ)

The search strategy (Figure 1) provided 356 articles from the Medline database, and 1700 documents were retrieved through the Google search engine. After manual removal of duplicate references, 104 articles were retrieved. Thirty of these did not contain pertinent patient data and were, therefore, excluded. The end result was 74 articles with 103 case reports containing detailed and pertinent individual patient data mostly retrieved through Medline[3-59], while 22.9% of these came from Google alone[60-76]. The individual patient data collected from the three patients treated for perforated midgut diverticulitis at Vestfold Hospital were added to this group, making a total of 106 patients (48 men and 58 women). The mean age of the group was 72.2 ± 13.1 years, and the age distribution was not normal ($P = 0.001$). The mean age for men was 73.7 ± 12.2 years and the mean age for women was 71.1 ± 13.8 years, with non-normal distribution for both sexes, $P = 0.005$ and $P = 0.001$, respectively. Sex distribution according to age was not significantly different in this group ($P = 0.296$). Patient age or sex did not have an impact on outcome ($P = 0.057$ and $P = 0.771$, respectively).

Preoperative assessment was described as plain radi-

Table 1 Overview of complications in Group Ⅰ (case reports)

Complications	Frequency	Present
General[1]	5	4.7
Surgical[2]	8	7.5
Both	3	2.9
None	59	55.7
Not available	31	29.2
Total	106	100

[1]Acute myocardial infarction, pulmonary embolism, pneumonia, deep vein thrombosis, urinary tract infection and stroke; [2]wound infection/dehiscence, anastomotic leakage, small bowel obstruction, intra-abdominal abscess and incisional hernia.

ography in 49 patients (53.3%) or CT in 70 (76.1%) in a total of 92 (86.8%) patients. Case reports did not include preoperative assessment in 14 (13.2%) patients. Where CT was performed, the correct preoperative diagnosis was made in 54 (77.1%) patients, while the probability of detecting perforated midgut diverticulitis without CT was 5.6% (two patients), both patients were previously known to have midgut diverticulosis. The difference in diagnosis with/without CT was statistically significant ($P = 0.001$).

Data on the duration of symptoms prior to hospitalization were present for 88 (83.0%) patients. The mean duration of symptoms prior to hospitalization was 3.6 d (range: 1-35 d). A longer duration of symptoms did not result in a poor outcome ($P = 0.748$). Data on fever were listed in 49 (46.2%) patients. Patients most often presented with moderate fever 23 (46.9%), while no fever or high fever was present in 26.5% each. Leukocyte and CRP levels were 13.8 ± 3.6 × 10^9/L and 155.4 ± 100.6 mg/L, respectively. Ninety-two (86.8%) patients underwent surgery, and 14 (13.2%) received conservative treatment. The surgical procedure most often performed was small bowel resection (83, 90.1%), followed by suture closure (5, 5.5%). Two patients (2.2%) underwent complex procedures that included multiple resections and 2 (2.2%) underwent surgical exploration with drainage. Access was through open surgery in 85 (92.4%) patients, while laparoscopic access was chosen in 5 (5.5%) and converted in 3 (60%). Two procedures (2.2%) were completed through laparoscopy. Data on postoperative complications were accessible in 73 patients. Complications occurred in 19.2% (Table 1). Fifteen (16.3%) patients underwent reoperation.

The operative report contained data on the distance of the perforated diverticulum to the ligament of Treitz in 29 patients, setting the mean distance to 41.7 ± 28.1 cm (range: 4-110 cm). Data on peritonitis was missing in five (4.7%) patients. There was no peritonitis or abscess in 30 (29.7%) patients, local peritonitis was present in 48 (47.5%), and 23 (22.8%) had diffuse peritonitis. Two (2.0%) patients had a mesenteric abscess. A reasonable correlation between the grade of peritonitis and reoperation rate was noted ($r = 0.43$, Spearman's test).

Seventeen (16.8%) patients had a solitary midgut diverticulum. Multiple jejunal diverticula were noted in 84 (83.2%) patients, while no data were provided for 5 (4.7%).

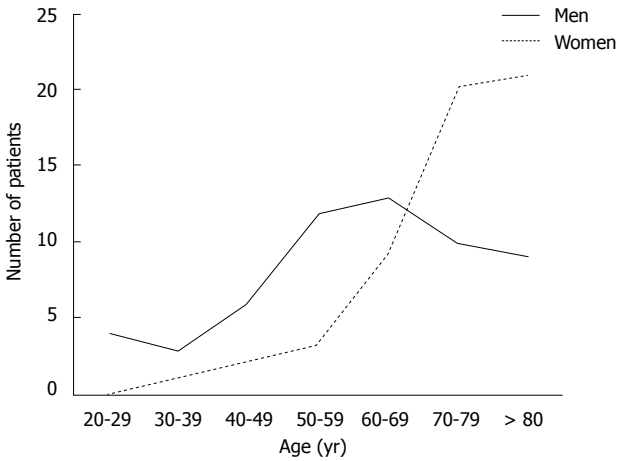


Figure 2 Correlation between sex and age for patients in the Norwegian patient registry.

Data on location and number of diverticula throughout the digestive tube were listed for 46 (43.4%) patients. Concomitant ileal diverticulosis was found in 18 (39.1%), while 31 (67.4%) had colon diverticulosis. In all, 21 (45.6%) patients had diverticulosis throughout the intestinal tract.

Mean hospital stay was 10.7 ± 7.9 d. Conservatively treated patients did not have a longer hospital stay than operated patients (10.6 ± 8.3 d *vs* 10.7 ± 7.9 d, respectively). Reoperated patients had a longer hospital stay 13.6 ± 8.4 d. There was a reasonable correlation between patient age and length of hospital stay ($r = 0.46$). Overall mortality was 5.7%. There was no statistically significant difference in outcomes for operated or nonoperated patients ($P = 0.814$).

Norwegian patient registry (Group Ⅱ)

The analysis of the Norwegian patient registry provided data on 113 patients, 57 men and 56 women. Patient mean age was 67.6 ± 16.4 years (range: 21-96 years). The age distribution within this group was not normal ($P = 0.04$). Mean age for men was 61.3 ± 16.2 years, while that for women was 74.7 ± 12.5 years, with non-normal distribution for both sexes, $P = 0.027$ and $P = 0.013$, respectively. The difference in age between the sexes was highly significant ($P = 0.001$) (Figure 2). The mean frequency of surgical procedures per year was 11.2 ± 0.9. The procedures most often performed were small bowel resection in 93 (82.3%) patients and enterorrhaphy in 17 (15%). Equivalent procedures performed through laparoscopic access were performed in 2 (1.8 %) and 1 (0.9 %) patients, respectively.

Historical controls (Group Ⅲ)

After the exclusion of patients under the age of 18 years, the group consisted of 47 patients (21 men and 26 women). Patient mean age was 65.4 ± 14.4 years. The age distribution within this group was normal ($P = 0.18$). Mean age for men was 61.5 ± 17.3 years, while that for women was 65.3 ± 14.4 years, with normal distribution for both sexes, $P = 0.29$ and $P = 0.76$, respectively.

Duration of symptoms prior to hospitalization was 6.9 d (range: 1-180 d). No patients had a preoperative diagnosis. Only one (2.1%) patient was treated conservatively, while the remaining 46 (97.9%) underwent surgery. Peroperative data were available for 24 (51.1%) patients, and cited as perforation in all, without grade of peritonitis. Twenty-three (48.9%) patients had data on number of diverticula, 18 (78.3%) had multiple and 5 (21.7%) had solitary diverticula. Small bowel resection was performed in 31 (67.4%) patients and suture closure in 15 (32.6%). Mortality in this group was 23.4%.

Comparison between patient groups

When length of history was concerned there was no difference between Groups I and Ⅲ (P = 0.241). Furthermore, there was no impact of length of history on survival between Groups I and Ⅲ (P = 0.198). No significant difference was detected in outcomes of conservative and surgical treatment (P = 0.23). However, there was a significant difference noted for type of surgical procedure, in favor of resection in Group Ⅰ compared to Group Ⅲ (P = 0.01), as well as the number of diverticula (P = 0.001). Mortality was significantly higher in Group Ⅲ compared to Group Ⅰ (P = 0.002).

DISCUSSION

One of the objective difficulties that a researcher encounters when investigating rare conditions is the absence of evidence and data. Data from the Norwegian patient registry show that a trial on a national level would prove to be difficult, including at most 10 patients per year. In these circumstances, one chooses the next best solution, reanalysis of small case series or case reports that contain sufficient individual patient data. A tendency to not publish case reports or small case series in indexed journals is noted, making this task even more demanding, hence, the contribution of the Google search engine in locating almost 20% of these case reports. We are well aware of the shortcomings of such a study; however, the use of abductive, probabilistic reasoning[77] allows for some valuable conclusions to be drawn.

The main finding of the present study is a transition in treatment, diagnosis and outcomes of patients suffering from perforated midgut diverticulitis. There seems to be a shift towards conservative treatment when properly diagnosed, a much higher percentage of accurate diagnosis, and small bowel resection is usually performed with lower mortality rates. This implies that elderly patients suffering from acute abdominal symptoms with elevated infection parameters should undergo a CT scan. Hence, the surgeon should have a high level of suspicion that the patient is suffering from perforated midgut diverticulitis after the initial CT scan is performed. Moreover, it seems that a substantial number of patients that could have been treated conservatively underwent surgery, because the majority of operated patients suffered from contained perforation. Surgical treatment should be avoided not only because of the burden of age and comorbidity in this patient group, but rather to avoid the risk of extensive resection in patients with multiple diverticula[1,2,78]. The fact that laparoscopy was so seldom used is even more surprising, because laparoscopic exploration and evaluation do not represent advanced minimally invasive surgical procedures.

The present study provides an opportunity to recommend the intensive use of laparoscopic exploration and evaluation in the emergency surgical setting. Nowadays, it seems that laparoscopic exploration provides at least as useful evaluation of abdominal pathology as open surgery does. Apart from the obvious benefits, laparoscopic exploration allows us to abandon the abdomen after lavage and drainage, and continue a conservative course[79]. A heavy proximal (within 1 m from the Treitz ligament) midgut mesentery with fibrin deposits is typical for the condition, and if generalized peritonitis is not present, our results seem to show that a conservative approach is feasible. The rationale is the fact that length of history prior to surgery does not seem to affect treatment outcome; there was no increase in mortality or morbidity between the groups. Even length of hospital stay was equal between patients operated upon and those conservatively treated. On the other hand, generalized peritonitis had a significant correlation with length of hospitalization and mortality, and should be treated surgically. The affected loop can be exteriorized through a mini-laparotomy after laparoscopic mobilization, and limited resection performed. Suture closure does not seem to have a place in the treatment of perforated midgut diverticulitis.

Such an approach would minimize the frequency of midgut resections in this high-risk population, while allowing the correct choice of patients for conservative treatment. A similar approach has recently had an impact on the treatment of sigmoid diverticulitis. Laparoscopic lavage and drainage have been advocated for generalized peritonitis due to perforated sigmoid diverticulitis. In this study, only 8% of patients underwent conversion and surgical resection, while the vast majority were handled conservatively after lavage and drainage[79].

It seems that correct diagnosis, together with the recognition of the immunological and physiological advantages of minimally invasive surgery, modern antibiotics and critical care could contribute to even better outcomes.

In summary, technological developments have led to better diagnosis of patients with perforated midgut diverticulitis. However, these have as yet not been applied in the operating room, where open surgery is still dominant over laparoscopy. When properly diagnosed, a transition in treatment is noticed towards conservative treatment. Only generalized peritonitis seems to have a significant correlation with length of hospitalization and mortality, and should be treated surgically. In these patients, laparoscopy and small bowel resection should be performed.

COMMENTS

Background

Perforated midgut diverticulitis is an uncommon, acquired condition that is only

sporadically observed and treated in surgical departments. Most of the literature consists of case reports, and this could be the reason for the confusion that still exists when clinical manifestations, diagnosis and treatment options are concerned. In addition, developments in surgery, radiology and other branches of medicine have occurred since the last review that has changed both surgical and conservative treatment.

Research frontiers

As demonstrated in the results this is a condition occurring in the elderly, and therefore has its challenges in choice of conservative treatment over surgical in the individual patient. Minimally invasive surgery would seem to have a place in this setting as well as the development of innovative surgical techniques to tackle this specific setting.

Innovations and breakthroughs

A transition to conservative treatment due to a correct preoperative diagnosis is apparent. Laparotomy continues to be dominant and the use of minimally access surgery is negligible. Small bowel resection is most often performed; suture closure is abandoned.

Applications

All elderly patients presenting with fever, infection and abdominal pain should have a computed tomography (CT) scan at admission. Surgery should be considered only in cases with generalized peritonitis. Conservative treatment should have a main role in treatment. Innovative minimally invasive procedures are required.

Terminology

Midgut is a segment of bowel that extends from the apex of the duodenal loop to the last third of the transverse colon. The midgut is supplied with blood by the superior mesenteric artery and innervated by the vagus nerve.

Peer review

The paper described a transition in diagnosis, treatment (a shift toward conservative treatment when properly diagnosed with CT), and outcomes (lower mortality rates) of patients suffering from perforated midgut diverticulitis. This topic will be of interest to WJG readers.

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BRIEF ARTICLE

Primary biliary cirrhosis-specific autoantibodies in first degree relatives of Greek primary biliary cirrhosis patients

Theodoros A Zografos, Nikolaos Gatselis, Kalliopi Zachou, Christos Liaskos, Stella Gabeta, George K Koukoulis, George N Dalekos

Theodoros A Zografos, Nikolaos Gatselis, Kalliopi Zachou, Christos Liaskos, Stella Gabeta, George N Dalekos, Department of Medicine and Research Laboratory of Internal Medicine, Medical School, University of Thessaly, 41110 Larissa, Greece
George K Koukoulis, Department of Pathology, Medical School, University of Thessaly, 41110 Larissa, Greece
Author contributions: Zografos TA, Zachou K and Dalekos GN had the original idea for the study, designed the study protocol and wrote the paper; Zografos TA, Gatselis N, Liaskos C and Gabeta S performed the whole immune serology work-up and along with Zachou K and Dalekos GN assessed the subjects from the immunological point of view; Koukoulis GK interpreted the histological data; Zografos TA, Zachou K and Gatselis N collected the data and performed the statistical analysis, and contributed to the final version of the paper; Koukoulis GK and Dalekos GN wrote the final version of the paper; and all authors approved the final draft of the paper.
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Correspondence to: George N Dalekos, MD, PhD, Department of Medicine and Research Laboratory of Internal Medicine, School of Medicine, University of Thessaly, Biopolis, 41110 Larissa, Greece. dalekos@med.uth.gr
Telephone: +30-241-3202285 Fax: +30-241-3501557
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Abstract

AIM: To determine the prevalence and significance of primary biliary cirrhosis (PBC)-specific autoantibodies in first-degree relatives (FDRs) of Greek PBC patients.

METHODS: The presence of antimitochondrial antibodies (AMA) and PBC-specific antinuclear antibodies (ANA) were determined using indirect immunofluorescence assays, dot-blot assays, and molecularly based enzyme-linked immunosorbent assays in 101 asymptomatic for liver-related symptoms FDRs of 44 PBC patients. In order to specify our results, the same investigation was performed in 40 healthy controls and in a disease control group consisting of 40 asymptomatic for liver-related symptoms FDRs of patients with other autoimmune liver diseases namely, autoimmune hepatitis-1 or primary sclerosing cholangitis (AIH-1/PSC).

RESULTS: AMA positivity was observed in 19 (only 4 with abnormal liver function tests) FDRs of PBC patients and none of the healthy controls. The prevalence of AMA was significantly higher in FDRs of PBC patients than in AIH-1/PSC FDRs and healthy controls [18.8%, 95% confidence interval (CI): 12%-28.1% *vs* 2.5%, 95% CI: 0.1%-14.7%, $P = 0.01$; 18.8%, 95% CI: 12%-28.1% *vs* 0%, 95% CI: 0%-10.9%, $P = 0.003$, respectively]. PBC-specific ANA positivity was observed in only one FDR from a PSC patient. Multivariate analysis showed that having a proband with PBC independently associated with AMA positivity (odds ratio: 11.24, 95% CI: 1.27-25.34, $P = 0.03$) whereas among the investigated comorbidities and risk factors, a positive past history for urinary tract infections (UTI) was also independently associated with AMA detection in FDRs of PBC patients (odds ratio: 3.92, 95% CI: 1.25-12.35, $P = 0.02$).

CONCLUSION: In FDRs of Greek PBC patients, AMA prevalence is significantly increased and independently associated with past UTI. PBC-specific ANA were not detected in anyone of PBC FDRs.

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Key words: Primary biliary cirrhosis; Antimitochondrial antibodies; Anti-gp210; Anti-sp100; Liver autoimmunity

Peer reviewers: Pietro Invernizzi, MD, PhD, Division of Internal Medicine and Hepatobiliary Immunopathology Unit, IRCCS Istituto Clinico Humanitas, Via A. Manzoni 113, 20089 Rozzano, Milan, Italy; Hans L Tillmann, Professor, Medizinische

Klinik und Poliklinik II, University Leipzig, Philipp Rosenthal, Str. 27, 04103 Leipzig, Germany

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INTRODUCTION

Primary biliary cirrhosis (PBC) is an autoimmune cholestatic liver disease of unknown cause which predominantly affects women and is characterized by immune-mediated destruction of the intrahepatic bile ducts, gradually leading to fibrosis, cirrhosis, and eventually, liver failure[1].

The disease is now diagnosed earlier than it was in the past as attested by the fact that more than 60% of PBC patients are asymptomatic at diagnosis as opposed to fewer than 40% a decade ago[2]. Early detection of PBC seems important, since patients with PBC, albeit asymptomatic, have reduced overall survival compared to controls[3], whereas timely treatment with ursodeoxycholic acid before the development of late-stage disease may normalize life expectancy[4,5].

The pathogenesis of PBC remains elusive, although it is generally considered to involve a combination of genetic and environmental factors[6]. The observed differences in PBC prevalence between populations living in different geographical regions but sharing similar genetic backgrounds support that environmental factors play a pivotal role in disease etiology[7,8]. Epidemiologic evidence suggests several non-genetic risk factors to be associated with PBC, including a history of urinary tract infections (UTI), past smoking and use of hormone replacement therapy[9]. Additionally, experimental data have proposed xenobiotics and several infectious agents, such as *Escherichia coli* and *Novosphingobium aromaticivorans* as initiating factors in the development of PBC[10].

Conversely, genetic factors also play an important role in conferring susceptibility to PBC, as indicated by the high concordance rate among monozygotic twins and the increased prevalence of PBC and other autoimmune disorders in patients' families[11,12]. During the past 15 years, several studies have reported familial PBC prevalence to range between 1.3% and 9%[13-18]. In addition to clustering of PBC and other autoimmune diseases in the families of PBC patients, the presence of antimitochondrial antibodies (AMA) aggregates in first-degree relatives (FDRs) of afflicted individuals as well, with AMA prevalence ranging between 1% and 13.1%[16,19-23]. Circulating AMA against the 2-oxo-acid dehydrogonase components of inner mitochondrial multi-enzyme complexes are considered the serological hallmark of PBC[1,24]. The main antigenic targets of AMA include the E2 subunits of the pyruvate dehydrogonase complex (PDC-E2), the branched-chain 2-oxo-acid dehydrogonase complex (BCOADC-E2), the oxoglutarate dehydrogenase complex (OGDC-E2), and the E1a subunit and the E3 binding protein of PDC[24]. AMA are present in 90%-95% of patients and are often detectable years before clinical signs appear[1,24]. Indeed, AMA detection in otherwise asymptomatic individuals with normal liver function tests may be associated with PBC-compatible histology in 40% of cases and eventual progression to clinically apparent disease[25,26].

In addition to AMA, previous studies have shown that antinuclear antibodies (ANA) giving a multiple nuclear dot (MND) or a rim-lime/membranous (RL/M) pattern by indirect immunofluorescence (IIF) are found in up to 65% of PBC patients if IgG isotype specific antibodies are investigated[27-29]. These antibodies seem to be disease specific (ANA-PBC specific) and have prognostic significance[27-29]. However, to the best of our knowledge these antibodies have not yet been investigated in asymptomatic for liver-related symptoms FDRs of PBC patients.

Accordingly, the aim of the present study was to determine the prevalence of AMA and ANA-PBC specific antibodies in asymptomatic for liver-related symptoms FDRs of Greek patients with PBC in comparison with asymptomatic FDRs from patients with other autoimmune liver diseases (disease control group) and healthy controls. To the best of our knowledge a combination of sensitive diagnostic techniques was used for the first time in order to improve the overall performance. In addition, we investigated the association of these antibodies with known comorbidities and reported risk factors for PBC as similar data on these issues is scarce in FDRs of PBC patients.

MATERIALS AND METHODS

Study population

From a cohort of 140 well-defined Greek PBC patients, 44 gave informed consent to participate in the study. Upon consent, patients were asked to provide information about their FDRs, i.e. parents, siblings and children. Subsequently, FDRs were contacted and were asked to participate in the study by attending an interview, physical examination and donation of blood. Accordingly, 101 FDRs of these 44 PBC patients were finally investigated. The diagnosis of PBC was based on the criteria published in our previous reports[30-33]. In brief, PBC patients met the following criteria: (1) positivity for AMA (positive titre $\geqslant$ 1/40) either by IIF on in-house fresh rodent tissue substrates confirmed by Western blot using in-house mitochondrial subtraction of rat livers, or by enhanced performance M2 enzyme-linked immunosorbent assay [M2 EP (MIT3) ELISA, QUANTA LiteR, INOVA Diagnostics, San Diego, CA], which was shown to have higher sensitivity compared to the conventional anti-M2[33]; (2) elevated cholestatic enzymes for more than 6 mo; and (3) histologic lesions consistent with PBC.

The disease control group consisted of 40 FDRs of Greek patients with well-defined autoimmune hepatitis-type 1 (AIH-1) or primary sclerosing cholangitis (PSC)

who were recruited through an identical process after an informed consent[34]. Their corresponding probands were 11 AIH-1 patients and 7 PSC patients.

All FDRs from the PBC patients and disease control patients underwent medical history and physical examination by a single investigator. The medical history was based on the US National Health and Nutrition Examination Survey questionnaire (NHANES 1999-2000)[35] with special attention to the environmental, familial and medical factors previously reported to associate with PBC[9]. Therefore, the questionnaire included data regarding demographics, smoking and alcohol consumption and past history of the FDRs. In particular, a past history of autoimmune diseases and other comorbidities reported to have increased prevalence in patients with PBC like Sjogren's syndrome, Hashimoto's thyroiditis, *etc*. was recorded in detail. From each subject serum samples were obtained and stored at -80 ℃, until assayed. Finally, a healthy control group consisted of 40 age and sex matched blood donors were also investigated. The study protocol was approved by the Ethical Committee of the Larissa Medical School, University of Thessaly, Larissa, Greece.

Detection of autoantibodies

IIF-Rodent tissues: AMA were detected by IIF on an in-house rat multiorgan substrate panel that included kidney, liver, and stomach using standard protocols[36-38]. The initial screening dilution was 1/40, and positive samples were further diluted in order to determine the maximum titer. Anti-total human Ig-fluorescein isothiocyanate conjugate was used (IgG, A, M, κ and λ; Dako Ltd.; High Wycombe, Bucks, United Kingdom) according to our laboratory practice[24,31,36]. AMA titers > 1/40 were considered positive.

IIF-HEp-2cells: A commercially available IIF assay was used for the semi-quantitative determination of ANA (NOVA Lite HEp-2, INOVA Diagnostics, San Diego, CA, positive titer > 1/40).

Dot blot assay: In order to confirm AMA-reactivity and to detect other liver-related autoantibodies, all sera were further examined using a commercial line (dot) blot assay (Euroline Liver Profile, Euroimmun AG, Lübeck, Germany), according to manufacturer's instructions. The Euroline Profile assay consists of membrane strips coated with thin parallel lines of purified, biochemically characterized antigens, allowing the detection of AMA, antibodies against liver kidney microsomes type 1, antibodies against liver cytosol type 1, and antibodies against soluble liver antigen/liver-pancreas antigen.

M2 ELISA: IgG-isotype specific AMA detection was also performed with an enhanced performance M2 ELISA, which utilizes a recombinant antigen (MIT3) containing the three immunodominant mitochondrial antigenic epitopes, namely PDC-E2, BCOADC-E2 and OGDC-E2 (INOVA Diagnostics, San Diego, CA), as we have described previously[33].

Utilizing all methods for the detection of AMA we have calculated the cumulative AMA reactivity of the study population, in which serum samples were considered positive if they fulfilled one of the following criteria: (1) they were AMA positive by IIF on rat multiorgan substrate at a dilution > 1/40 accompanied by AMA reactivity either in the dot blot assay or the enhanced MIT3-based ELISA; and (2) they were AMA positive on M2 MIT3-based ELISA irrespective of the AMA titer obtained by the IIF assay.

Anti-sp100 and anti-gp210 ELISAs: Semi-quantitative detection of anti-sp100 and anti-gp210 antibodies of the IgG class was performed with commercially available specific ELISAs according to the manufacturer's instructions (QUANTA Lite sp100 and gp210, INOVA Diagnostics, San Diego, CA). Serum samples were tested in duplicate and considered positive for PBC-specific ANA only when fulfilled the following criteria: MND or RL/M reactivity by IIF on Hep2 cells accompanied by positivity against anti-sp100 or anti-gp210, respectively, by using the molecularly based assays mentioned above (ELISAs)[24].

Statistical analysis

Data normality was analyzed using the Kolmogorov-Smirnov test. Continuous, normally distributed variables are presented as mean ± SD, and non-normally distributed variables as median (interquartile range). Categorical and ordinal data are presented as frequencies and/or percentages. Student's *t*-test, Mann-Whitney *U*-test, Kruskal-Wallis test, χ^2 test, Fischer's exact test and binary logistic regression analysis were also used, where applicable. *P* values of < 0.05 were considered statistically significant.

RESULTS

Demographic characteristics

The baseline demographic characteristics of the study population are summarized in Table 1. In total, the FDRs group consisted of 141 participants (61% children, 24.1% siblings and 14.9% parents).

Since a significant percentage of the FDRs group consisted of descendants of patients with autoimmune liver diseases, the mean age in the proband group was higher than the FDRs group. However, this difference was statistically significant only in PBC probands compared to PBC FDRs (58.8 ± 11.8 *vs* 40.8 ± 17.9, $P < 0.01$). Moreover, there were no statistically significant differences in age between the PBC FDRs, the AIH-1 FDRs and the PSC FDRs group.

Clinical disorders in FDRs

The prevalence of clinical disorders reported by FDRs of patients with autoimmune liver diseases is shown in Table 2. There were no statistically significant differences

Table 1 Demographic characteristics of the study population

	Total	Male	Female	Age (yr)
PBC probands	44	4 (9.1)	40 (90.9)	58.8 ± 11.8
PBC FDRs	101	45 (44.6)	56 (55.4)	40.8 ± 17.9
Parents	9 (8.9)	5 (55.6)	4 (44.4)	72.8 ± 8.8
Children	69 (68.3)	30 (43.5)	39 (56.5)	31.3 ± 10.6
Siblings	23 (22.8)	10 (43.5)	13 (56.5)	56.7 ± 10.7
AIH-1 probands	11	2 (18.2)	9 (81.8)	48.8 ± 22.0
AIH-1 FDRs	21	12 (57.1)	9 (42.9)	37.2 ± 14.0
Parents	3 (14.3)	1 (33.3)	2 (66.7)	46.0 ± 19.3
Children	13 (61.9)	7 (53.8)	6 (46.2)	36.6 ± 10.3
Siblings	5 (23.8)	4 (80)	1 (20)	33.6 ± 20.0
PSC probands	7	4 (57.1)	3 (42.9)	40.7 ± 9.5
PSC FDRs	19	9 (47.4)	10 (52.6)	49.9 ± 21.0
Parents	9 (47.4)	4 (44.4)	5 (55.6)	67.4 ± 9.4
Children	4 (21.1)	2 (50)	2 (50)	17.8 ± 2.2
Siblings	6 (31.6)	3 (50)	3 (50)	45.0 ± 6.1
Healthy controls	40	17 (42.5)	23 (57.5)	43.1 ± 9.8

Data are shown as *n* (%) or mean ± SD. PBC: Primary biliary cirrhosis; AIH-1: Autoimmune hepatitis-1; PSC: Primary sclerosing cholangitis; FDRs: First-degree relatives.

Table 2 Surgical history and comorbidities in first degree relatives of patients with autoimmune liver diseases and healthy controls *n* (%)

	PBC FDRs (*n* = 101)	AIH-1 and PSC FDRs (*n* = 40)	Healthy (*n* = 40)
History of comorbidities			
Sjögren's syndrome	7 (6.9)	1 (2.5)	0
Raynaud's syndrome	8 (7.9)	2 (5)	0
Autoimmune thyroiditis	4 (3)	0	0
Rheumatoid arthritis	1 (1)	0	0
Systemic lupus erythematosous	0	1 (1)	0
Asthma	3 (3)	4 (10)	0
Atopic dermatitis	2 (2)	1 (2.5)	0
Vitiligo	1 (1)	0	0
Psoriasis vulgaris	1 (1)	0	0
Erythema nodosum	1 (1)	0	0
Type 1 diabetes mellitus	1 (1)	1 (2.5)	0
Idiopathic thrombocytopenic purpura	1 (1)	0	0
Osteoporosis	4 (4)	3 (7.5)	0
Urinary tract infections	33 (32.7)[a]	11 (27.5)	6 (15)
Hypercholesterolemia	10 (9.9)	7 (17.5)	4 (10)
Hypertension	6 (5.9)	5 (12.5)	3 (7.5)
History of surgery			
Tonsillectomy	14 (13.9)	3 (7.5)	3 (7.5)
Appendectomy	18 (17.8)	7 (17.5)	5 (12.5)
Cholecystectomy	6 (5.9)	1 (2.5)	1 (2.5)
Inguinal hernia	14 (13.9)[b,c]	0	1 (2.5)

[a]$P < 0.05$ *vs* healthy controls; [b]$P = 0.01$ *vs* autoimmune hepatitis-1 (AIH-1)/primary sclerosing cholangitis (PSC) first-degree relatives (FDRs); [c]$P = 0.05$ *vs* healthy controls. PBC: Primary biliary cirrhosis.

between FDRs of patients with PBC and FDRs of patients with AIH-1 or PSC, with the exception of a higher reported prevalence of inguinal hernia and subsequent surgical repair in PBC FDRs (Table 2). Of note, PBC FDRs reported significantly higher prevalence of UTI compared to healthy controls [32.7%, 95% confidence interval (CI): 23.8%-42.8% *vs* 15%, 95% CI: 6.3%-30.5%, $P < 0.05$] (Table 2). Binary logistic regression analysis demonstrated that only a past history of UTI was independently associated with PBC FDRs (odds ratio: 2.89, 95% CI: 1.1-7.6, $P = 0.03$).

Table 3 Autoantibody prevalence in first degree relatives of patients with primary biliary cirrhosis, compared to autoimmune hepatitis-1 and primary sclerosing cholangitis first degree relatives and healthy controls *n* (%)

Autoantibody	PBC FDRs (*n* = 101)	AIH-1 and PSC FDRs (*n* = 40)	Healthy controls (*n* = 40)	P^1	P^2
AMA (IIF)	10 (9.9)	1 (2.5)	0	NS	< 0.001
1:80	8 (7.9)	1 (2.5)	-		
1:160	1 (1)	-	-		
1:320	1 (1)	-	-		
IgG MIT3-based ELISA	13 (12.9)	0	0	0.02	0.02
AMA positivity[3]	19 (18.8)	1 (2.5)	0	0.01	0.003
RL/M pattern	8 (7.9)	1 (2.5)	0	NS	NS
1:80	8 (7.9)	1 (2.5)	-		
MND pattern	9 (8.9)	5 (12.5)	0	NS	0.06
1:80	7 (6.9)	3 (7.5)	-		
1:160	1 (1.0)	-	-		
1:320	-	2 (5.0)	-		
1:640	1 (1.0)	-	-		
PBC-specific ANA[3] positivity	0	1 (2.5)	0	NS	NS

[1]Comparison between primary biliary cirrhosis (PBC) first degree relatives (FDRs) and autoimmune hepatitis-1 or primary sclerosing cholangitis (AIH-1/PSC) FDRs; [2]comparison between PBC FDRs and healthy controls; [3]for definition of antimitochondrial antibodies (AMA) and PBC-specific antinuclear antibodies (ANA) positivity please see text (materials and methods section). IIF: Indirect immunofluorescence; ELISA: Enzyme-linked immunosorbent assay; RL/M: Rim-lime/membranous; MND: Multiple nuclear dot; NS: Not significant.

Autoantibody prevalence in FDRs

According to the above-mentioned criteria, AMA were detected in 20 FDRs of patients with autoimmune liver diseases (14.2%, 95% CI: 9.1%-21.3%) and none of the healthy controls (0%, 95% CI: 0%-10.9%, $P = 0.008$). The prevalence of AMA was also significantly higher in FDRs of patients with PBC compared to FDRs of patients with AIH-1 or PSC (18.8%, 95% CI: 12%-28.1% *vs* 2.5%, 95% CI: 0.1%-14.7%, $P = 0.01$; Table 3). PBC-FDRs had significantly increased AMA prevalence compared to healthy controls (18.8%, 95% CI: 12%-28.1% *vs* 0%, 95% CI: 0%-10.9%, $P = 0.003$).

ANA reactivity on Hep-2 cells is presented in Table 3. Specifically, antibodies giving RL/M pattern of ANA were observed in 7.9% (95% CI: 3.7%-15.5%) of PBC-FDRs. RL/M pattern of ANA was also observed in one PSC-FDR and in none of the controls; however, this difference did not approach significance. MND pattern of ANA tended to be detected in more AIH-1/PSC-FDRs (12.5%, 95% CI: 4.7%-27.6%) and PBC-FDRs (8.9%, 95% CI: 4.4%-16.7%) compared to none of healthy controls ($P = 0.05$ and $P = 0.06$, respectively). According to the predefined criteria of the study, PBC-specific ANA positivity was only observed in one FDR of a PSC patient.

Table 4 Characteristics of first degree relatives according to antimitochondrial antibodies positivity

	AMA positive (n = 20)	AMA negative (n = 121)	P
Demographics			
Female	12 (60)	63 (52.1)	NS
Age (yr)	47.8 ± 17.8	40.4 ± 18.0	NS
Proband disease (PBC)	19 (95)	87 (67.8)	0.012
Educational level, %			NS
Primary	41.70	26.50	
Secondary	33.30	39.30	
Higher	25	34.20	
Body mass index (kg/m^2)	25.7 ± 3.9	25.7 ± 5.0	NS
Smoking and alcohol consumption			
Smoking	8 (40)	30 (27.5)	NS
Daily alcohol intake (g)	19.8 ± 38.2	17.7 ± 43.5	NS
Medical history			
Urinary tract infection	11 (55)	33 (27.3)	0.01
Number of UTI events (range)	4 (1-5)	1 (1-2.5)	NS
Sicca symptoms	4 (20)	19 (15.7)	NS
Autoimmune disorders	3 (15)	5 (4.1)	NS
Autoimmune thyroid disease	2 (10)	2 (1.7)	NS
Diabetes	1 (5)	3 (2.5)	NS
Osteoporosis	0 (0)	7 (5.8)	NS
Tonsillectomy	4 (20)	13 (10.7)	NS
Appendectomy	3 (15)	22 (18.2)	NS
Inguinal hernia	2 (10)	12 (9.9)	NS
Laboratory values (U/L)			
AST	29.6 ± 11.4	23.6 ± 9.7	NS
ALT	32.6 ± 25.5	26.1 ± 26.4	NS
γ-GT	29.6 ± 24.6	28.9 ± 37.7	NS
ALP	76.0 ± 23.3	73.5 ± 35.7	NS

Data are shown as n (%) or mean ± SD. UTI: Urinary tract infections; AMA: Antimitochondrial antibodies; PBC: Primary biliary cirrhosis; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GT: Gamma-glutamyl transpeptidase; ALP: Alkaline aminophosphatase; NS: Not significant.

FDR characteristics associated with AMA positivity

Demographic and clinical characteristics according to the presence of AMA are presented in Table 4. AMA-positive FDRs were predominantly female (60%). Age, level of education, smoking habits, body mass index, and daily alcohol consumption were not significantly associated with AMA-positivity. However, a medical history of UTI was significantly more frequently reported in AMA-positive FDRs, compared to AMA-negative FDRs, whereas a trend towards a higher number of UTI recurrences in AMA-positive FDRs was observed (Table 4). Binary logistic regression revealed having a proband with PBC or a positive history for UTI were both independently associated with AMA positivity (odds ratio: 11.24, 95% CI: 1.27-25.34, P = 0.03 and odds ratio: 3.92, 95% CI: 1.25-12.35, P = 0.02, respectively).

Clinical and laboratory manifestations of PBC in AMA-positive FDRs

Of the 20 FDRs, who were determined positive for AMA reactivity, 5 (25%) presented with abnormal liver enzymes and their characteristics are shown in Table 5. Male FDRs with AMA were asymptomatic either for liver- or extrahepatic-related symptoms whereas one female suffered from Sicca syndrome and two from Hashimoto thyroiditis. Physical examination was unremarkable in all 5 subjects. To date, none of these subjects have agreed to undergo liver biopsy.

Table 5 Characteristics of antimitochondrial antibodies-positive relatives with abnormal liver enzymes

	Age (yr)	Proband disease	Relationship to the proband	AST/ ALT (U/L)	ALP/ γ-GT (U/L)	Symptoms
Patient A	44	PBC	Son	37/45	102/122	No
Patient B	47	PBC	Brother	23/18	65/104	No
Patient C	40	PBC	Son	37/90	67/154	No
Patient D	24	PBC	Daughter	60/165	136/61	Sicca + Hashimoto's thyroiditis
Patient E	52	PSC	Sister	53/103	91/94	Hashimoto's thyroiditis

AST: Aspartate aminotransferase (upper limit: 35 U/L); ALT: Alanine aminotransferase (upper limit: 40 U/L); γ-GT: Gamma-glutamyl transpeptidase (upper limit: 37 U/L); ALP: Alkaline aminophosphatase (upper limit: 104 U/L); PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis.

DISCUSSION

The data reported herein demonstrate that FDRs of patients with PBC have increased prevalence of AMA compared to healthy controls, whereas the presence of PBC-specific ANA was similar among all groups. During the seventies, two studies investigating the autoantibody prevalence in relatives of patients with PBC reported AMA detection in approximately 7.5% of the screened subjects[19,20]. In a subsequent study, Caldwell *et al*[21] reported a somewhat lower prevalence of 4.9% in 41 FDRs of 27 PBC probands. Using IIF, Floreani *et al*[16] observed AMA positivity in 6.1% of 115 relatives of unspecified relationship to 30 PBC probands. Conversely, in a 2004 study from Brazil, AMA prevalence in FDRs was estimated to be as low as 1%[22]. Taken together, these studies illustrate that AMA prevalence in PBC FDRs varies significantly according to the method used for AMA detection and their prevalence in the general population from which the study population is derived. Indeed, several studies from Europe have reported AMA prevalence in the general population to range between 0.5% and 0.9%, while in studies from China and Japan it was lower (range: 0.16% to 0.64%), demonstrating again slight variances according to methodology used and the origin of the populations studied[39-42].

In the most comprehensive study concerning AMA prevalence in FDRs of PBC patients conducted in Minnesota, an area with one of the highest reported PBC prevalence[43], Lazaridis *et al*[23] found AMA positivity in 13.1% of the FDRs and 1% of healthy controls. AMA prevalence in FDRs of PBC patients in our study was found to be slightly higher (18.8%) compared to that recorded in all the abovementioned reports[16,19-23]. In particular, taking into account that first, the proportion of female FDRs

in the study of Lazaridis *et al*[23] was higher (64%-68.5%) compared to the present study, and second, that their population consisted predominantly of older FDRs, the actual difference in AMA prevalence in FDRs of Greek and US patients with PBC might be even higher.

One possible explanation for the higher AMA prevalence in FDRs of Greek PBC patients compared to the previous reports[16,19-23] is that we used a combination of diagnostic techniques for AMA detection which may improve the overall sensitivity. Indeed, to the best of our knowledge this is the first study to utilize a combination of IIF, MIT3-based ELISA and dot-blot for the detection of AMA in FDRs of PBC patients. It is well known that almost 90% of AMA-positive PBC sera react to PDC-E2, while approximately 10% of them react only to BCOADC-E2 and/or OGDC-E2 indicating that, as we have previously demonstrated, testing for AMA by the first generation commercial M2-ELISAs (using only the PDC-E2 as the primary substrate) or using only IIF would result in low sensitivity of AMA testing[33]. The latter has been shown recently by our group since up to 12.6% of AMA-negative PBC patients by IIF were MIT3-ELISA positive whereas 4.8% of MIT-3 negative PBC sera were AMA positive by IIF[33].

An alternative explanation for the relatively high rates of AMA positivity in FDRs of Greek PBC patients observed in our study could be attributed to the background of the study population. Although no published data on PBC prevalence exist for Greece, subjects of Greek descent have been shown to carry the highest prevalence of PBC in an Australian study[7]. Of course, the participation of a relatively low number of patients' families (44 families out of 140 patients) may have led to selection bias, which theoretically could influence our analysis.

Of interest, AMA prevalence was significantly higher in FDRs reporting a past history of UTI with a trend towards a higher number of UTI recurrences in AMA-positive FDRs. This finding was stable even after multivariate analysis suggesting UTI as an independent factor for AMA detection among FDRs of PBC patients. Epidemiological data have suggested past history of UTI as a risk factor for PBC development[9] whereas patients with recurrent UTI have an increased incidence of AMA seropositivity[44]. Recurrent UTI have been suggested to increase the exposure to microbial antigens like *Escherichia coli* which may then induce the development of PBC in genetically susceptible individuals through molecular mimicry mechanisms[45].

The second major finding of this study is that FDRs of Greek patients with PBC do not have increased prevalence of PBC-specific ANA. Using HEp2 cells as substrate, ANA in PBC patients are heterogeneous[24,46]; however, ANA presenting with the MND or rim-like membranous (RLM) pattern by IIF have proven to be clinically significant for PBC[24,46]. The molecular targets of ANA with the MND pattern in PBC are mainly the nuclear body protein sp100 and less often the promyelocytic leukemia protein, small ubiquitin-related modifiers and more recently SP140 antigen, whereas the molecular targets of the RLM pattern are structural components of the nuclear envelope primarily gp210, nucleoporin p62 and lamin B receptor[24,46-48]. A possible limitation of our study is that we have determined reactivities only against the two most common molecular targets of PBC-specific ANA, namely anti-gp210 and anti-sp100, which are highly specific for PBC and have been found in up to 65% of PBC patients if IgG-isotype specific antibodies (IgG1, 2, 3 and 4) are investigated[24,27-29,46,49].

According to the available evidence, there are significant differences between AMA- and ANA-reactivity in patients with PBC, since AMA may appear several years before the clinical or histological signs of disease, whereas PBC-specific ANA are more frequently found in those patients with advanced disease[24,27,28,46,50]. Moreover, the detection of PBC-specific ANA seems to confer prognostic information, because when ANAs become detectable during the course of disease, a higher rate of disease progression could be expected[24,46,49,51,52]. Therefore, the lack of PBC-specific ANA in AMA-positive FDRs might suggest a pre-clinical stage of the disease. Furthermore, the detection of PBC-specific ANA in FDRs who exhibit AMA-positivity may indicate further diagnostic evaluation. According to a cohort study by Invernizzi *et al*[52], antibodies against the nuclear pore complex (anti-NPCs), may aid prognostic evaluation in patients presenting at early PBC stages, i.e., those with normal total bilirubin levels at presentation. Actually, the contribution of anti-NPC detection in a prognostic model incorporating the Mayo score and the histological stage was greater in early PBC cases.

In conclusion, using a combination of tests for AMA detection, we have provided evidence that AMA prevalence in asymptomatic for liver-related symptoms FDRs of Greek patients with PBC is significantly increased compared to FDRs of Greek patients with other autoimmune liver diseases and healthy controls. This prevalence is in fact the highest reported to date among FDRs of PBC patients and it is significantly associated with past history of UTI. Further large-scale multicentre prospective studies with the use of combination techniques for AMA detection are needed in order to precisely define the AMA prevalence in FDRs from PBC patients and assess whether these subjects will develop the clinical and biochemical spectrum of PBC in the future. We are currently undertaking such follow-up in our AMA positive FDRs from PBC patients. PBC-specific ANA are not detected in FDRs of PBC patients. Nevertheless, in our opinion the sequential determination of the latter antibodies as a marker of progression from a pre-clinical to an early clinical stage should be further evaluated.

COMMENTS

Background

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized usually by an insidious or even silent onset with long asymptomatic course which can eventually lead to cirrhosis and liver failure. The laboratory hallmark of the disease is the detection of antimitochondrial antibodies (AMA). Early diagnosis is crucial for timely treatment and improvement of the quality of life and

survival. Among several factors, genetic predisposition is important in conferring susceptibility to PBC. Indeed, during the past 15-20 years, several studies have reported familial clustering of PBC whereas the presence of AMA seems to aggregate in first-degree relatives (FDRs) of afflicted individuals as well, with AMA prevalence among FDRs ranging between 1% and 13.1%.

Research frontiers

The research hotspot is to estimate the prevalence and associations of AMA along with PBC-specific ANA with known risk factors of PBC development in asymptomatic FDRs of Greek patients with PBC. This could be very important in an attempt to identify new asymptomatic PBC cases and potentially alter the disease course by the prompt initiation of the indicated therapy.

Innovations and breakthroughs

In the present study, the authors estimated the prevalence and significance of AMA in FDRs of PBC patients using for the first time a combination set of sensitive diagnostic techniques (indirect immunofluorescence, dot-blot assays, and molecularly based enzyme-linked immunosorbent assays) in order to improve the overall performance of AMA detection. In addition, they assessed for the first time, the significance of PBC-specific ANA in the same population since these autoantibodies are known prognostic factors for PBC outcome and progression.

Applications

Based on their results the authors believe that further large-scale multicentre prospective studies with the use of combination techniques for AMA detection (not only indirect immunofluorescence) are needed in order to precisely define the AMA prevalence in FDRs of PBC patients and assess whether these subjects will develop the clinical and biochemical spectrum of PBC or not in the future.

Terminology

PBC is an autoimmune cholestatic liver disease of unknown etiology with clustering in families of PBC patients whereas AMA detection is higher in FDRs of PBC patients compared to healthy population. The use of combination tests for AMA detection in FDRs of PBC patients seems to be the design of choice in order to identify asymptomatic individuals who potentially will develop the disease since the present study showed the highest incidence of AMA in FDRs of PBC ever reported using this methodology. This is of particular importance especially among those with a past history of urinary tract infection.

Peer review

This study described the prevalence and significance of AMA and PBC-specific ANA in FDRs of Greek PBC patients. The authors concluded that AMA prevalence in this group of subjects is significantly increased compared to healthy and FDRs of patients with other autoimmune liver diseases like autoimmune hepatitis-1 or primary sclerosing cholangitis. In addition, AMA detection in FDRs of PBC patients was independently associated with past history of urinary tract infections. To the contrary, PBC-specific ANA were not detected in PBC FDRs. Therefore, the study provides significant data, which is of great importance, as it includes original and useful data for these otherwise healthy subjects (FDRs of PBC patients).

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BRIEF ARTICLE

Titanium dioxide induced inflammation in the small intestine

Carolina Maciel Nogueira, Walter Mendes de Azevedo, Maria Lucia Zaidan Dagli, Sérgio Hiroshi Toma, André Zonetti de Arruda Leite, Maria Laura Lordello, Iêda Nishitokukado, Carmen Lúcia Ortiz-Agostinho, Maria Irma Seixas Duarte, Marcelo Alves Ferreira, Aytan Miranda Sipahi

Carolina Maciel Nogueira, André Zonetti de Arruda Leite, Maria Laura Lordello, Iêda Nishitokukado, Carmen Lúcia Ortiz-Agostinho, Aytan Miranda Sipahi, Department of Gastroenterology, Laboratory of Clinical and Experimental Gastroenterology, School of Medicine, University of São Paulo, Cerqueira César, 01246-900 São Paulo, Brazil
Walter Mendes de Azevedo, Department of Fundamental Chemistry, Federal University of Pernambuco, Cidade Universitária, 50670-901 Recife, Brazil
Maria Lucia Zaidan Dagli, Department of Pathology, Veterinary Medicine School, University of São Paulo, Cidade Universitária, 05508-270 São Paulo, Brazil
Sérgio Hiroshi Toma, Laboratory of Supramolecular Chemistry and Nanotechnology, Chemistry Institute, University of São Paulo, Cidade Universitária, 05513-970 São Paulo, Brazil
Maria Irma Seixas Duarte, Laboratory of Transmissible Disease, Medicine School, University of São Paulo, Cerqueira César, 01246-900 São Paulo, Brazil
Marcelo Alves Ferreira, Laboratory of Cellular Biology, Medicine School, University of São Paulo, Cerqueira César, 01246-900 São Paulo, Brazil
Author contributions: Nogueira CM is the main researcher to perform the present study; de Azevedo WM synthetized TiO_2 nanoparticles; Dagli MLZ carried out histopathological evaluation of tissue; Toma SH helped and supported in all matters related to micro- and nanoparticles providing information on how to store and handle them as well as on how to prevent particle aggregation before their use in animals, and also determined the size and phase of particles; Duarte MIS helped standardize immunohistochemistry reactions; Leite AZA helped standardize the experimental protocol and most of the experiments; Lordello ML, Nishitokukado I, Ortiz-Agostinho CL helped in most of the experiments; Ferreira MA provided the transmission eletron mycroscopy images of the particles; and Sipahi AM designed the study.
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Correspondence to: Dr. Aytan Miranda Sipahi, Department of Gastroenterology, Laboratory of Clinical and Experimental Gastroenterology (LIM 07), School of Medicine, University of São Paulo, Av. Dr. Arnaldo 455, Cerqueira César, 01246-900 São Paulo, Brazil. aytan@usp.br
Telephone: +55-11-30617279 Fax: +55-11-30617279
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Abstract

AIM: To investigate the effects of titanium dioxide (TiO_2) nanoparticles ($NPTiO_2$) and microparticles ($MPTiO_2$) on the inflammatory response in the small intestine of mice.

METHODS: Bl 57/6 male mice received distilled water suspensions containing TiO_2 (100 mg/kg body weight) as $NPTiO_2$ (66 nm), or $MPTiO_2$ (260 nm) by gavage for 10 d, once a day; the control group received only distilled water. At the end of the treatment the duodenum, jejunum and ileum were extracted for assessment of cytokines, inflammatory cells and titanium content. The cytokines interleukin (IL)-1b, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, IL-23, tumor necrosis factor-α (TNF-α), intracellular interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) were evaluated by enzyme-linked immunosorbent assay in segments of jejunum and ileum (mucosa and underlying muscular tissue). $CD4^+$ and $CD8^+$ T cells, natural killer cells, and dendritic cells were evaluated in duodenum, jejunum and ileum samples fixed in 10% formalin by immunohistochemistry. The titanium content was determined by inductively coupled plasma atomic emission spectrometry.

RESULTS: We found increased levels of T $CD4^+$ cells (cells/mm^2) in duodenum: NP 1240 ± 139.4, MP 1070 ± 154.7 *vs* 458 ± 50.39 ($P < 0.01$); jejunum: NP 908.4 ± 130.3, MP 813.8 ± 103.8 *vs* 526.6 ± 61.43 ($P < 0.05$); and ileum: NP 818.60 ± 123.0, MP 640.1 ± 32.75 *vs* 466.9 ± 22.4 ($P < 0.05$). In comparison to the control group, the groups receiving TiO_2 showed a statistically significant increase in the levels of the inflammatory cytokines IL-12, IL-4, IL-23, TNF-α, IFN-γ and TGF-β. The cytokine production was more pronounced in the ileum (mean ± SE): IL-12: NP 33.98 ± 11.76, MP 74.11 ± 25.65 *vs* 19.06 ± 3.92 ($P < 0.05$); IL-4: NP 17.36 ± 9.96, MP 22.94 ± 7.47 *vs* 2.19 ± 0.65 ($P < 0.05$); IL-23: NP 157.20 ± 75.80, MP 134.50 ± 38.31 *vs* 22.34 ± 5.81 ($P < 0.05$); TNFα: NP 3.71 ± 1.33, MP 5.44 ± 1.67 *vs* 0.99

± 019 ($P < 0.05$); IFNγ: NP 15.85 ± 9.99, MP 34.08 ± 11.44 *vs* 2.81 ± 0.69 ($P < 0.05$); and TGF-β: NP 780.70 ± 318.50, MP 1409.00 ± 502.20 *vs* 205.50 ± 63.93 ($P < 0.05$).

CONCLUSION: Our findings indicate that TiO_2 particles induce a Th1-mediated inflammatory response in the small bowel in mice.

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Key words: Titanium dioxide; Microparticles; Nanoparticles; Immune response; Small intestine; Mice

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INTRODUCTION

We are exposed daily through inhalation, ingestion or contact to many environmental and engineered particles. The gastrointestinal tract is continuously exposed to particles in the diet, such as titanium dioxide (TiO_2), which is an insoluble white powder that is used as an anti-caking and whitening agent in many commercial products, including paint, cosmetics, plastics, paper, pharmaceuticals, and food colorants[1]. The ingestion of exogenous particles is substantial, as demonstrated by Lomer *et al*[2], who reported that about 40 mg of exogenous particles are ingested per person per day in the United Kingdom. Besides the amount of exogenous particles that are ingested, nanoparticulates can be inhaled and trapped on the mucus of the respiratory tract and then swallowed, thus reaching the gastrointestinal tract[3]. Clearance studies in animals involving radiolabeled nanoparticles (NP) showed that 30%-50% of inhaled NP are translocated to the gastrointestinal tract[4].

TiO_2 can be found as microparticles (MP) (diameter of 0.1 to 1.0 μm) and NP (diameter less than 100 nm). The increasing utilization of nanomaterials in industrial as well as consumer products has increased the possibility of human exposure but their influence on human health remains uncertain. Although studies reporting TiO_2 toxicity are still limited, some suggest that smaller particles produce a greater inflammatory response in comparison with the larger fine-sized particles of similar chemistry at the same mass concentrations[5-7].

Many studies have revealed that exposure to TiO_2 can cause adverse effects such as the generation of reactive oxygen species[8-10], inflammatory responses[5,6,11-13], tumors[14], cytotoxicity[15] and apoptosis[16]. *In vivo* studies showed that NP can be accumulated in many organs such as the liver, kidney, spleen, lung, heart and brain[17,18], thus generating a number of adverse effects. Previous investigations have found that TiO_2 accumulates in the intestine in rats[19] and fish[20] and migrates to other organs. Accumulation of TiO_2 inside the intestinal cells, especially in lymphoid-rich areas (Peyer's patch), might lead to damaging outcomes such as inflammation and could be involved in the pathogenesis of inflammatory bowel disease[21,22]. However, little is known about the influence of either micro- or NP on the gut, which is potentially exposed to particles in the diet, such as TiO_2. To date, most of the studies regarding the adverse effects of TiO_2 particles on human health have involved the pulmonary tract. No available *in vivo* work has evaluated the impacts of TiO_2 particles in terms of their inflammatory potential within the gastrointestinal tract.

Therefore, the present study was designed to investigate the effects of TiO_2 as MP and as NP on the inflammatory response in the small intestine of mice. We aimed to evaluate cytokine production and inflammatory cell proliferation in the small intestine of mice after oral exposure to TiO_2.

MATERIALS AND METHODS

Particles

Uncoated anatase TiO_2 microparticles ($MPTiO_2$) (260 nm) that are commercially available for use in food, pharmaceuticals, and cosmetics were obtained from Evonik Degussa (Kronos® 1171). Uncoated TiO_2 nanoparticles ($NPTiO_2$) (mean diameter of 66 nm), consisting mostly of anatase, were synthesized by Professor de Azevedo WM from the Department of Fundamental Chemistry of the Federal University of Pernambuco (Recife, Brazil) at pH = 2.0, followed by centrifugation. Particle size was determined by dynamic light scattering Nanotrac® (Microtrac Inc., United States) by Professor Toma SH from the Laboratory of Supramolecular Chemistry and Nanotechnology of the Chemistry Institute of the University of São Paulo (São Paulo, Brazil). Particle phase was characterized using an X-ray diffractometer Rigaku Miniflex® (Rigaku Corporation, Japan) under monochromatic radiation, Cu Kα (1.541 Å, 30 kV, 15 mA, 0.02°, 2° to 61° range), also by Professor Toma SH.

Animals and treatment

Bl 57/6 male mice (20 to 25 g) were obtained from the Center of Bioterism of the School of Medicine, University of São Paulo (São Paulo, Brazil). Animals were housed in cages in a ventilated room in a 12-h light/dark cycle. Food and water were available ad libitum. They were acclimated to this environment for 1 wk before treatment. All animal experimental procedures were in

compliance with the School of Medicine, University of São Paulo Ethics Committee. Mice were randomly divided into three groups of 12 animals, and received either distilled water suspensions containing TiO_2 (100 mg/kg body weight) as MP, or as NP, or distilled water as a control. The suspension was given by gavage for 10 d, once a day. TiO_2 particles were suspended in 500 μL of distilled water. The suspension was mixed and sonicated immediately before being administered to animals to minimize particle aggregation. At the end of the treatment the animals were weighed and killed in a CO_2 chamber, and had their duodenum, jejunum and ileum extracted for assessment of cytokines, inflammatory cells and titanium content.

Assessment of cytokines

Segments of jejunum and ileum - mucosa and underlying muscular tissue - were extracted from animals, stored at -80 ℃ and subsequently homogenized with Tris-buffer (10 mmol)-ethylenediamine tetraacetic acid (1 mmol)-Triton (1%) containing protease, aprotinin, chymostatin and leupeptin inhibitors (1 μg/mL of solution) and phenylmethylsulfonyl fluoride (1 μL/mL of solution). After homogenization, the sample was centrifuged at 14 000 *g* for 10 min at 4 ℃ and the supernatant was stored at -80 ℃ until the cytokines were analyzed using an enzyme-linked immunosorbent assay (ELISA). Interleukin-1β (IL-1β) IL-4, IL-6, IL-8 (Keratinocyte Chemoattractant), IL-10, IL-12, IL-13, IL-17, IL-23, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) were evaluated using ELISA kits (IL-1β, IL-4, IL-6, IL-12, IL-13, IL-17, IL-23, TNF-α, IFN-γ, and TGF-β from eBioscience; and IL-10 and IL-8 from R and D systems) according to the manufacturer's recommendations.

Quantification of inflammatory cells

Duodenum, jejunum and ileum samples were fixed in 10% formalin and were embedded in paraffin. Five-mm thick sections were cut for evaluation of $CD4^+$ and $CD8^+$ T cells, natural killer cells (CD57), and dendritic cells (S100+) by immunohistochemistry using specific antibodies: anti-CD4 [Dako M0834 (OPD4 clone)], anti-CD8 [Dako® M7103 (clone C8/144B)], anti-S100+ [Dako® M7103 (clone C8/144B)], and anti-CD57 [Dako® M7103 (clone C8/144B)]. For cell quantification, the observers randomly selected ten areas and counted positive cells under the 40 × objective.

Histopathological evaluation of small intestine

Duodenum, jejunum and ileum samples were fixed in 10% formalin and embedded in paraffin. Five-mm thick sections were cut, placed onto glass slides and then stained with HE. The slides were examined by optical microscopy (E800, Nikon, United States) by Professor Dagli MLZ from the Department of Pathology of the School of Veterinary Medicine, University of São Paulo (São Paulo, Brazil).

Titanium content analysis

The titanium content in intestinal samples from each group was analyzed with the purpose of verifying the efficacy of the treatment, and to make sure that the animals of the control group did not contain titanium (Ti) in their tissues. To determine the presence of Ti in the small intestine of the animals receiving TiO_2 particles, the experiment was repeated including two animals in each group. At the end of the treatment, the small intestine was extracted for analysis of the titanium content by inductively coupled plasma atomic emission spectrometry (ICP-AES). The small intestine was removed in its entirety (from the pylorus to the ileocecal valve), preserving the mucosa and muscle tissue, stored at -80 ℃ and taken to the Basic Analysis Laboratory of the Analytical Center, Chemistry Institute, University of São Paulo (São Paulo, Brazil) where it was homogenized and processed by the local team following their standard protocol.

Statistical analysis

Data for each parameter evaluated are shown as the mean ± SE. Data were analyzed assuming a gamma distribution (identity link) and using generalized linear models. Pairwise comparisons and Fisher's least significant difference post-hoc test were applied to evaluate the differences between the groups. $P < 0.05$ was considered significant. Commercially available software was used for analysis (PASW Statistics Version 18, United States).

RESULTS

Titanium content

The titanium content in the small intestine from each group was determined by ICP-AES. The control group showed no detectable levels of titanium in the intestine. The titanium content (mg/kg of tissue) in the intestine was (animal 1/animal 2): 1.43/11.68 in the NP group, and 0.39/0.22 in the MP group.

Cytokine concentration in jejunum and ileum

In comparison to the control group, $MPTiO_2$ and $NPTiO_2$ generated a statistically significant increase in inflammatory cytokines. The group receiving $MPTiO_2$ showed an enhanced concentration of IFN-γ and IL-23 in the jejunum, and IL-12, TNF-α, IFN-γ, IL-4, IL-23, and TGF-β in the ileum (Figure 1). The group receiving $NPTiO_2$ showed increased TNF-α, IFN-γ, IL-4, IL-23, and TGF-β, but only in the ileum. There was no significant difference between the groups receiving $MPTiO_2$ and $NPTiO_2$, as both showed statistically similar levels of cytokine production. A more important cytokine production was found in the ileum compared to the jejunum.

Inflammatory cell infiltration in small intestine

We found a statistically significant increase in T $CD4^+$ cells in the duodenum, jejunum and ileum of mice treated with $MPTiO_2$ and $NPTiO_2$, compared to the control group. There was no significant difference between the

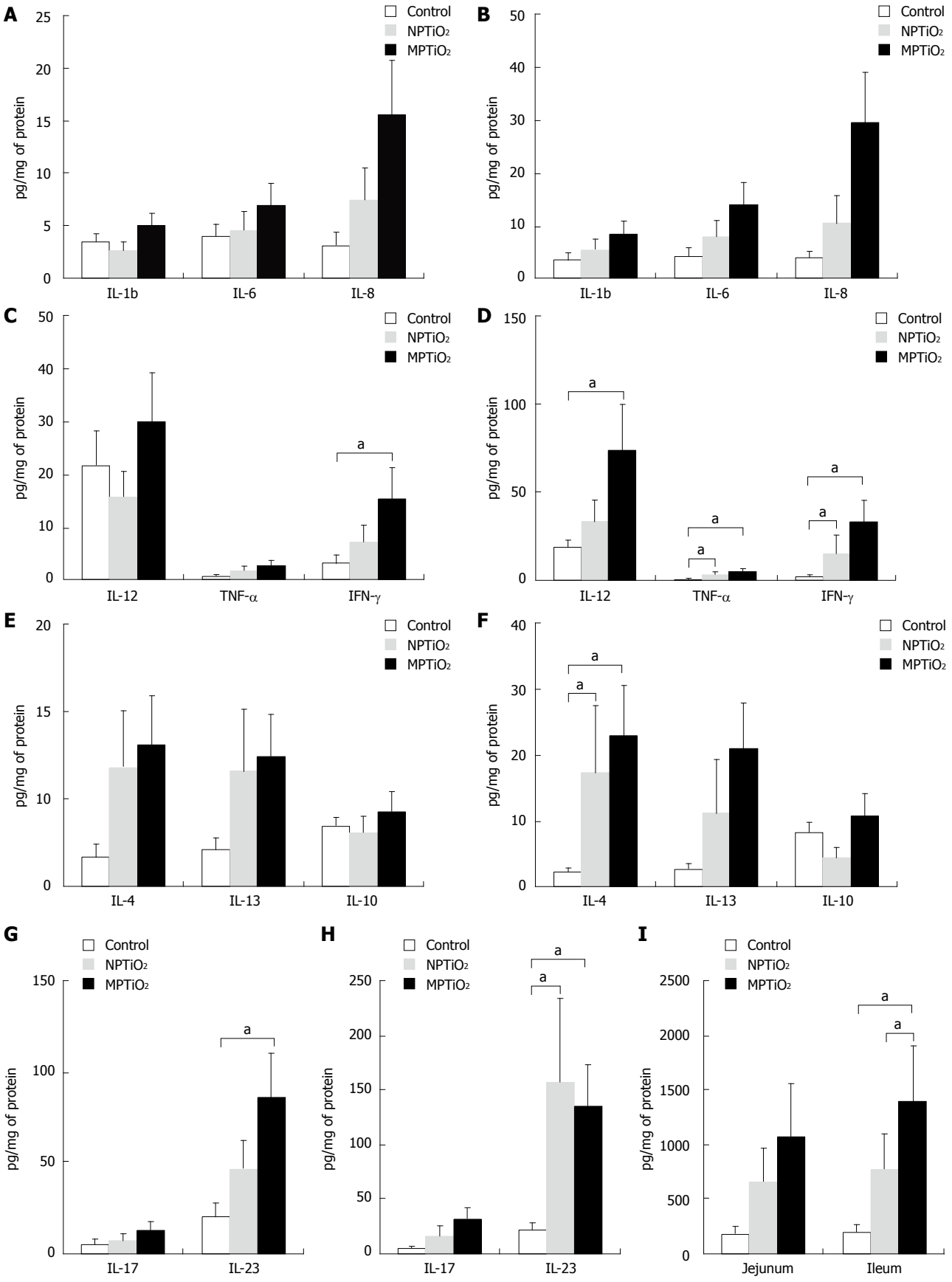


Figure 1 Mean and SE of cytokine concentration in the small intestine of mice according to treatment groups. A: Pro-inflammatory cytokines in the jejunum; B: Pro-inflammatory cytokines in the ileum; C: T-helper (Th) 1 type cytokines in the jejunum; D: Th1 type cytokines in the ileum; E: Th2 type cytokines in the jejunum; F: Th2 type cytokines in the ileum; G: Th17 type cytokines in the jejunum; H: Th17 type cytokines in the ileum; I: Transforming growth factor-β in the jejunum and ileum. $^aP < 0.05$ for pairwise comparison test. $NPTiO_2$: Titanium dioxide nanoparticles; $MPTiO_2$: Titanium dioxide microparticles; IL: Interleukin; IFN: Intracellular interferon.

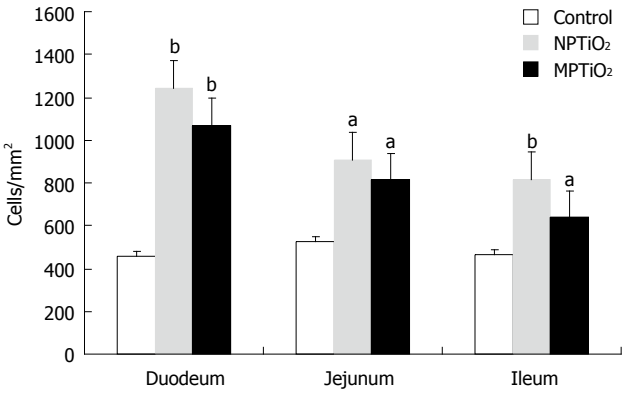


Figure 2 T CD4$^+$ cells quantified by immunohistochemistry in the duodenum, jejunum and ileum of mice, according to treatment group. Mean and SE of $^aP < 0.05$, $^bP < 0.01$ *vs* control group for pairwise comparison test. $NPTiO_2$: Titanium dioxide nanoparticles; $MPTiO_2$: Titanium dioxide microparticles.

$MPTiO_2$ and $NPTiO_2$ groups. The results are illustrated as the mean and SE in Figure 2. Mice treated with $MPTiO_2$ or $NPTiO_2$ showed no increase in T CD8$^+$, natural killers, or dendritic cells in the small intestine (data not shown).

Histopathological evaluation of the small intestine

No major histological changes were found in small intestine samples of experimental animals, except for hypertrophy and hyperplasia of the mucosal epithelium of mice receiving TiO_2 particles, which were not seen in the control group. These findings were observed in all three regions of the small intestine (duodenum, jejunum and ileum) of mice treated with $NPTiO_2$, while animals treated with $MPTiO_2$ showed these effects only in the ileum.

DISCUSSION

Inflammatory response

To date, there have been few data regarding the inflammatory potential of TiO_2 particles in the intestine. Here, we sought to evaluate inflammatory responses induced by TiO_2 in the small intestine in mice. We found that TiO_2 as micro- and nano-sized particles produced a pro-inflammatory response in the small intestine by generating increased inflammatory cytokine production and T CD4$^+$ cell proliferation. The generation of pro-inflammatory cytokines and T CD4$^+$ cells induced by $NPTiO_2$ was also described by Schanen *et al*[23] in an *in vivo* immune construct study.

The main cytokines enhanced in our study were IL-12, IFN-γ, TNF-α, IL-4, IL-23 and TGF-β. IL-12, TNF-α and IFN-γ are Th1-type cytokines, whereas IL-23 and TGF-β are both involved in the Th17 pathway. In murine models, TGF-β together with IL-6 promotes differentiation of naïve T cells to Th17 cells[24]. Here we found no significant increase in IL-6 or IL-17, which is produced by Th17 cells. Taken together, these data suggest that TiO_2 particles provoked a pro-inflammatory response mainly through the Th1-mediated pathway in the small bowel in mice. Other authors found a Th-2 mediated immune response in the lungs, induced by nano-sized TiO_2 exposure in mice[13,25]. However, inflammatory responses differ depending on the organ, and thus the immune response caused by exposure to TiO_2 may diverge between the respiratory tract and the gut.

Cytokine production was more pronounced in the ileum. These findings might be related to differences concerning particle uptake throughout the gut. It is known that the ileum presents the greatest concentration of M cells (Peyer's patch) in the intestine, which are believed to represent the main pathway of particle uptake across the gastrointestinal tract[26,27]. Li *et al*[28] observed greater absorption of lipid NP in the ileum and colon of rats when compared to other segments of the intestine, reinforcing the importance of M cells as a pathway of particle uptake. Given that the ileum represents the major site of particle uptake, we would expect to find a more substantial inflammatory response in this area.

We also evaluated histopathological changes in the small intestine of mice after exposure to TiO_2. We observed hypertrophy and hyperplasia of the mucosal epithelium in both groups receiving TiO_2 particles. These findings were also described by other authors in TiO_2-related studies. Alveolar epithelium hypertrophy was observed in the lungs of rats exposed to nano-sized TiO_2[29]. Mice, rats and hamsters showed histopathological changes consistent with alveolar epithelial cell hypertrophy and hyperplasia after long-term inhalation of fine TiO_2[30].

Taken together our data provide evidence that micro- and nano-sized TiO_2 particles induce a pro-inflammatory response in the small intestine in mice, after a short period of oral exposure.

The titanium content of the small intestine samples was determined by ICP-AES at the end of the experiments to guarantee the efficacy of the treatment with TiO_2 and to ensure that the control group had no detectable levels of titanium in their tissues. Our results demonstrated that TiO_2 particles were absorbed by the small intestine in mice after a short period of oral exposure, as the animals that received TiO_2 particles had titanium in their tissues at the end of the experiment. The control group showed no detectable levels of titanium in any sample. We found greater amounts of titanium in the small intestine of the animals receiving $NPTiO_2$ in comparison to those receiving $MPTiO_2$. These findings indicate that smaller particles may be absorbed to a greater extent than larger ones in the gut.

Nanoparticles vs microparticles

Previous investigations of TiO_2 particles found that TiO_2 as nano-sized particles is more toxic than similarly composed, but larger sized particles[5-7,11,29,31]. Thus, we aimed to compare the effects of micro- and nano-sized TiO_2 on the small intestine. However, we found no statistically significant difference in cytokine secretion or T CD4$^+$ cell proliferation between the groups who received $MPTiO_2$

and NPTiO_2. Other authors have already reported the lack of significant differences in the pulmonary effects in rats exposed to TiO_2 particles of different sizes[14,32-35].

In conclusion, We demonstrated that over a short period TiO_2 as micro- and nano-particles induced a Th1-mediated inflammatory response in the small intestine of mice, especially in the ileum. These findings provide evidence of the inflammatory potential of TiO_2 particles in the gastrointestinal tract. Since we are exposed to TiO_2 particles on a daily basis, as well as to many other engineered particles, these data should be taken into consideration when evaluating the safety of biomaterials.

COMMENTS

Background

Titanium dioxide (TiO_2) is a white pigment that is widely found as nano- and micro-sized particles added to commercial products such as food, drugs, cosmetics, *etc*. Many studies involving the respiratory tract warn of adverse effects resulting from exposure to TiO_2. Although the gastrointestinal tract is exposed to TiO_2 particles on a daily basis there is little information regarding their adverse effects on the intestine. Adverse effects resulting from exposure to TiO_2, such as cytotoxicity, generations of reactive oxygen species, tumors, inflammation, allergic reactions, lung emphysema, among others, have been reported in studies involving the respiratory tract. Although the lung is the most widely studied organ as far as exposure to particles such as TiO_2 is concerned, the gastrointestinal tract is considerably more exposed to environmental particles.

Research frontiers

The present study shows that nano- and micro-sized particles may lead to an inflammatory process in the bowel. As a result, small particles of TiO_2, which are present in the diet, may be involved in the development of inflammatory bowel disease.

Innovations and breakthroughs

Most researches related to TiO_2 particles studied their effects on the respiratory tract or were performed *in vitro*. This study was conducted *in vivo* to investigate the effects of TiO_2 as micro- and nanoparticles on the inflammatory response in the intestine of mice. To do so, mice received high doses of TiO_2 by gavage for 10 d and subsequently the small intestine was extracted for assessment of cytokines, infiltration of inflammatory cells and morphological alterations of tissue. Authors found the highest concentration of cytokines [interleukin (IL)-12, IL-4, IL-23, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) and of $CD4^+$ T cell in the intestine of animals that received TiO_2, suggesting the pro-inflammatory effect of these particles on the small intestine of mice.

Applications

Based on their results, authors can infer that TiO_2 elicited a pro-inflammatory response in the small intestine of mice, which seems to be predominantly a T-helper 1-type response and more pronounced in the ileum. These data are *in vivo* evidence of the inflammatory potential of TiO_2 particles on the gastrointestinal tract. Their findings emphasize the need to investigate the adverse effects of TiO_2 particles on health since they are exposed to products containing this substance on a daily basis.

Terminology

Microparticles are particles whose size ranges from 0.1 and 100 μm; Nanoparticles are particles smaller than 100 nm. Nanoscale materials are developed to display some specific features based on their size, shape, surface, *etc*. TiO_2 is a white powder that is used as an anti-caking and whitening agent in many commercial products, including paint, cosmetics, plastics, paper, pharmaceuticals and food colorants

Peer review

This study reports on the mice intestinal inflammatory responses to TiO_2 nano- and micro-particles administration. The presented results show that daily administration of 100 mg/kg of TiO_2 nano- as well as micro-particles elicited substantial inflammatory responses in the intestinal tissue and jejunum, as evidenced by the increase in IL-12, TNF-α, IFN-γ, IL-4, IL-23 and TGF-β. These effects were observed with both types of particles. Considering the increasing use of various form of titanium in the food industry and pharmaceuticals and implants, this adds an important evidence to a growing list of potential adverse effects of Ti on human health.

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BRIEF ARTICLE

"Extended" radical cholecystectomy for gallbladder cancer: Long-term outcomes, indications and limitations

Yoshio Shirai, Jun Sakata, Toshifumi Wakai, Taku Ohashi, Katsuyoshi Hatakeyama

Yoshio Shirai, Jun Sakata, Toshifumi Wakai, Taku Ohashi, Katsuyoshi Hatakeyama, Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata City 951-8510, Japan
Author contributions: Shirai Y conceived the study and drafted the manuscript; Sakata J helped to draft the manuscript and performed statistical analyses; Wakai T performed chart review and follow-up of the study cohort; Ohashi T helped with chart review and patient follow-up; Hatakeyama K was responsible for the whole study and participated in its coordination; and all authors read and approved the final manuscript.
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Correspondence to: Yoshio Shirai, MD, PhD, Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata City 951-8510, Japan. shiray@med.niigata-u.ac.jp
Telephone: +81-25-2272228 Fax: +81-25-2270779
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Abstract

AIM: To delineate indications and limitations for "extended" radical cholecystectomy for gallbladder cancer: a procedure which was instituted in our department in 1982.

METHODS: Of 145 patients who underwent a radical resection for gallbladder cancer from 1982 through 2006, 52 (36%) had an extended radical cholecystectomy, which involved *en bloc* resection of the gallbladder, gallbladder fossa, extrahepatic bile duct, and the regional lymph nodes (first- and second-echelon node groups). A retrospective analysis of the 52 patients was conducted including at least 5 years of follow up. Residual tumor status was judged as no residual tumor (R0) or microscopic/macroscopic residual tumor (R1-2). Pathological findings were documented according to the American Joint Committee on Cancer Cancer Staging Manual (7th edition).

RESULTS: The primary tumor was classified as pathological T1 (pT1) in 3 patients, pT2 in 36, pT3 in 12, and pT4 in 1. Twenty-three patients had lymph node metastases; 11 had a single positive node, 4 had two positive nodes, and 8 had three or more positive nodes. None of the three patients with pT1 tumors had nodal disease, whereas 23 of 49 (47%) with pT2 or more advanced tumors had nodal disease. One patient died during the hospital stay for definitive resection, giving an in-hospital mortality rate of 2%. Overall survival (OS) after extended radical cholecystectomy was 65% at 5 years and 53% at 10 years in all 52 patients. OS differed according to the pT classification ($P < 0.001$) and the nodal status ($P = 0.010$). All of 3 patients with pT1 tumors and most (29 of 36) patients with pT2 tumors survived for more than 5 years. Of 12 patients with pT3 tumors, 8 who had an R1-2 resection, distant metastasis, or extensive extrahepatic organ involvement died soon after resection. Of the remaining four pT3 patients who had localized hepatic spread through the gallbladder fossa and underwent an R0 resection, 2 survived for more than 5 years and another survived for 4 years and 2 mo. The only patient with pT4 tumor died of disease soon after resection. Among 23 node-positive patients, 11 survived for more than 5 years, and of these, 10 had a modest degree of nodal disease (one or two positive nodes).

CONCLUSION: Extended radical cholecystectomy is indicated for pT2 tumors and some pT3 tumors with localized hepatic invasion, provided that the regional nodal disease is limited to a modest degree (up to two positive nodes). Extensive pT3 disease, pT4 disease, or marked nodal disease appears to be beyond the scope of this radical procedure.

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Key words: Gallbladder neoplasms; Radical surgery; Hepatectomy; Bile duct resection; Lymph node excision; Prognosis

Peer reviewers: Abdul-Wahed Meshikhes, MD, Department of Surgery, King Fahad Specialist Hospital, Amir Bin Thabit St, Dammam 31444, Eastern Province, Saudi Arabia; Pankaj Garg, Dr., Consultant, Department of General Surgery, Fortis Super Speciality Hospital, Mohali, Punjab, Panchkula 134112, India

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INTRODUCTION

The history of radical surgery for gallbladder cancer started with cholecystectomy combined with wedge resection of the gallbladder fossa (without regional lymphadenectomy) in the early 20th century[1,2]. However, outcomes after this procedure were disappointing even in tumors limited to the wall of the gallbladder, probably due to the lack of regional lymphadenectomy[1,2]. In 1954, Glenn *et al*[3] first proposed a radical resection procedure with intended regional lymphadenectomy (portal lymph node dissection), designated as "radical cholecystectomy" (Glenn operation), for localized gallbladder cancer. Pack *et al*[4] in 1955 and Fahim *et al*[5] in 1962 advocated radical resection consisting of hepatectomy and portal lymph node dissection, and this strategy remains the one recommended by the National Comprehensive Cancer Network (NCCN) for pathological T1b (pT1b) or more advanced gallbladder cancer[6]. Outcomes after such radical resections, however, remain unsatisfactory, particularly for node-positive patients[7-10].

In Japan, an aggressive attitude toward gallbladder cancer emerged and gradually grew among specialized hepatobiliary surgeons in the 1970s and 1980s[11]. In this context, "extended" radical cholecystectomy (designated as the modified Glenn operation) was instituted in 1982 in our department (Figure 1)[11-13], as a modification of the radical cholecystectomy described by Glenn *et al*[3,14]. The procedure involves *en bloc* resection of the gallbladder, gallbladder fossa, extrahepatic bile duct, and the regional lymph nodes as defined previously[11,15]. Although this procedure has been widely used for gallbladder cancer among Japanese surgeons[11,16-18], indications and limitations remain to be established.

This study aimed to delineate indications and limitations for the extended radical cholecystectomy procedure for gallbladder cancer by retrospectively analyzing the long-term outcomes of 52 patients who underwent this procedure in the study department. The study goal was to definitively establish the role of this particular procedure in surgical management strategies for gallbladder cancer.

MATERIALS AND METHODS

Patient population

From May 1982 through December 2006, 145 consecutive patients underwent a variety of radical resection procedures for gallbladder cancer (including cancer arising in the cystic duct[19]) in the study department (Table 1). A radical resection was defined as an *en bloc* resection of both the primary tumor and the regional lymph nodes. This study period was selected in order to evaluate long-term outcomes after resection; the follow-up period was at least 5 years in all the patients. Of these, 52 (36%) who underwent an extended radical cholecystectomy (modified Glenn operation; Figure 1) were selected as our study cohort. They comprised 39 women and 13 men ranging in age from 43 to 84 years (median, 66.5 years).

Among the 52 patients, 34 underwent the extended radical cholecystectomy as an initial radical resection and 18 underwent this procedure as a radical second resection after a prior simple cholecystectomy for presumed benign disease. Although early (pT1) tumors do not warrant radical resection[20], three patients with pT1 tumor underwent the extended radical cholecystectomy because pT2 or more advanced disease was not ruled out before resection.

Scope of "extended" radical cholecystectomy

Extended radical cholecystectomy involves a cholecystectomy, wedge resection of the gallbladder fossa with a rim of non-neoplastic liver tissue (approximately 2 cm in thickness or more), resection of the suprapancreatic segment of the extrahepatic bile duct (bile duct resection), and regional lymph node dissection in an *en bloc* fashion (Figure 1). The main differences between this procedure and the original radical cholecystectomy described by Glenn *et al*[3,14] comprise the extent of regional lymphadenectomy and the presence or absence of bile duct resection.

The regional lymph nodes of the gallbladder include the cystic duct, pericholedochal, posterior superior (posterosuperior) pancreaticoduodenal, retroportal, right celiac, and hepatic artery node groups[11,15]. The cystic duct and pericholedochal node groups have been regarded as the first-echelon nodes of the gallbladder, whereas the other node groups as the second-echelon nodes[11,15]. Extended radical cholecystectomy harvests both the first- and second-echelon nodes *en bloc* (Figure 1).

We perform a bile duct resection to facilitate the regional lymphadenectomy, to remove pericholedochal lymphatic vessels and nodes simultaneously, to remove the possible presence of ductal (periductal) involvement[21], and to avoid the occurrence of ischemic biliary stricture after aggressive periductal nodal dissection[22].

The extent of hepatectomy appears to be similar between the extended procedure and the original described by Glenn *et al*[3,14]. With regard to resection of the gallbladder fossa for invasive tumors, we emphasize the need for complete excision of the cystic plate to avoid violating the subserosal tumor plane (Figure 2).

Table 1 Radical resection procedures for 145 patients with gallbladder cancer

Procedure	*n*
Extended cholecystectomy	
C + WR + BD + N[1]	52
C + WR + N	23
C[2] + BD + N	10
C[2] + N	9
More extensive resection	
C + H + BD + N	25
C + WR + PD + N	15
C + H + PD + N	9
C[2] + PD + N	2

[1]Designated as "extended" radical cholecystectomy (modified Glenn operation) since 1982[11]; [2]cholecystectomy with full-thickness dissection: cholecystectomy combined with removal of the cystic plate. C: Cholecystectomy; WR: Wedge resection of the gallbladder fossa; BD: Resection of the extrahepatic bile duct; N: Regional lymphadenectomy; H: Major hepatectomy defined as removal of two sections or more; PD: Whipple or pylorus-preserving pancreaticoduodenectomy.

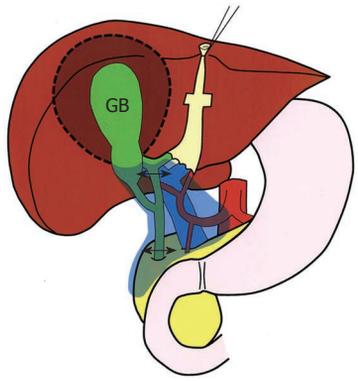


Figure 1 "Extended" radical cholecystectomy for gallbladder cancer. The dashed line indicates the scope of wedge resection of the gallbladder fossa (about 2 cm in thickness or more). The double-headed arrows indicate lines of division of the extrahepatic bile duct. The pale blue area indicates the extent of regional lymph node dissection, which achieves *en bloc* harvesting of both the first-echelon nodes (cystic duct and pericholedochal node groups) and the second-echelon nodes (posterosuperior pancreaticoduodenal, retroportal, right celiac, and hepatic artery node groups)[11,15]. GB: Gallbladder.

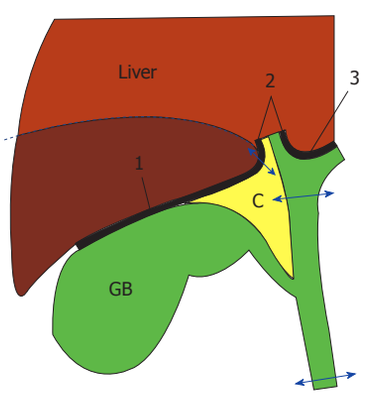


Figure 2 Wedge resection of the gallbladder fossa in "extended" radical cholecystectomy. The dashed line indicates the line of hepatic parenchymal division. The short double-headed arrow indicates the line of division of the cystic plate. The long double-headed arrows indicate lines of division of the extrahepatic bile duct. At the end of parenchymal division, the cystic plate is identified as a fibrous plate connecting with the right portal pedicle, and then divided at its base. Arabic numerals indicate the plate system of the liver (1: Cystic plate; 2: The dense fibrous sheath encircling the right portal pedicle; 3: Hilar plate). GB: Gallbladder; C: The triangle of Calot.

Among the 52 patients who underwent an extended radical cholecystectomy, 19 with suspected (or confirmed) regional nodal disease also underwent a dissection of the paraaortic lymph nodes (cephalad to the origin of the inferior mesenteric artery). Paraaortic nodal disease was classified as N2 disease according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (7th edition)[19].

Pathological examination

Immediately after resection, the surgeon(s) retrieved lymph nodes from the node-bearing adipose tissues of the fresh surgical specimen. A total of 871 regional lymph nodes (excluding paraaortic nodes) were retrieved from the 52 patients (median 16, range 6 to 36 per patient). Resected specimens together with the retrieved lymph nodes were submitted for histological examination. Residual tumor status was judged as no residual tumor (R0) or microscopic/macroscopic residual tumor (R1-2). All pathological findings were documented according to the AJCC Cancer Staging Manual (7th edition)[19].

Patient follow-up

Adjuvant chemotherapy was administered to 23 patients at the discretion of the individual surgeon. No patients received adjuvant radiotherapy. Patients discharged to home were followed regularly in outpatient clinics every 1-6 mo for at least 5 years, with a median follow-up period of 188 mo (range: 82-351 mo).

Prognostic factors after extended radical cholecystectomy

To determine prognostic factors after extended radical cholecystectomy, the following 15 conventional variables were tested univariately and multivariately in 52 patients who underwent the procedure (Table 2): age, gender, gallstone (absent *vs* present), timing of radical resection (initial resection *vs* second resection), adjuvant chemotherapy (absent *vs* present), size of the primary tumor (≤ 60 mm *vs* > 60 mm), pT classification, pathological regional lymph nodes (pN) classification, pathological distant metastasis (pM) classification, histological type, histological grade, lymphatic vessel invasion, venous invasion, perineural invasion, and residual tumor status.

Statistical analysis

Medical records and survival data were obtained for all 52 patients. Overall survival (OS) was defined as the interval between the date of definitive resection and the date of last follow up or death from any cause. The Kaplan-Meier method was used to estimate cumulative OS rates, and the log rank test was used to evaluate differences between groups. The Cox proportional hazards regression model using a step-backward fitting procedure was applied to

Table 2 Prognostic factors after "extended" radical cholecystectomy for 52 patients with gallbladder cancer

Variable	*n*	Survival rate (%)		Univariate analysis	Multivariate analysis	
		5-yr	10-yr	*P* value	Relative risk (95% CI)	*P* value
Age (yr)				0.002		
≤ 65	20	80	80			
> 65	32	50	35			
pT classification[1]				< 0.001		0.001
pT1 plus pT2	39	82	70		1.000	
pT3 plus pT4	13	15	0		5.947 (2.054-17.221)	
pN classification[1]				0.037		
pN0	29	79	67			
pN1	12	58	33			
pN2	11	36	36			
pM classification[1]				< 0.001		0.002
pM0	48	71	57		1.000	
pM1	4	0	0		20.068 (3.083-130.620)	
Histological type[1]				< 0.001		0.001
Adenocarcinoma	48	71	57		1.000	
Others	4	0	0		16.919 (3.015-94.939)	
Histological grade[1]				0.021		
G1	17	88	73			
G2 plus G3 plus G4	35	54	43			
Lymphatic vessel invasion (L)[1]				0.016		
L0	20	85	73			
L1	32	53	40			
Venous invasion (V)[1]				0.004		
V0	24	83	74			
V1	28	50	34			
Perineural invasion				0.041		0.084
Absent	36	72	62		1.000	
Present	16	50	31		2.396 (0.889-6.460)	
Residual tumor status[1]				< 0.001		0.071
R0	47	72	58		1.000	
R1 plus R2	5	0	0		3.585 (0.897-14.329)	

[1]According to the American Joint Committee on Cancer Cancer Staging Manual (7th edition)[19]. CI: Confidence interval; pT classification: Pathological T classification; pN classification: Pathological regional lymph nodes classification; pM classification: Pathological distant metastasis classification; G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated; G4: Undifferentiated; L0: No lymphatic vessel invasion; L1: Lymphatic vessel invasion; V0: No venous invasion; V1: Microscopic venous invasion; R0: No residual tumor; R1: Microscopic residual tumor; R2: Macroscopic residual tumor.

identify independent prognostic factors. In this model, a step-wise selection was used for variable selection with entry and removal limits of $P < 0.05$ and $P > 0.10$, respectively.

The IBM SPSS Statistics 19 software (IBM Japan, Tokyo, Japan) was used for all statistical evaluations. All tests were two-tailed, and $P < 0.05$ was taken to indicate statistical significance.

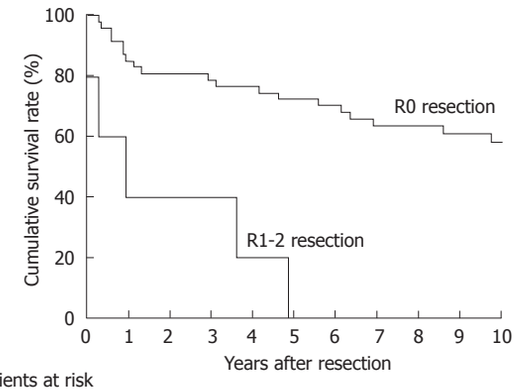
RESULTS

The pT was classified as pT1 in 3 patients, pT2 in 36, pT3 in 12, and pT4 in 1. Twenty-three patients had lymph node metastases, with the number of positive nodes per patient ranging from 1 to 41 (median, 2); 11 patients had a single positive node, 4 had two positive nodes, and 8 had three or more positive nodes. None of the three patients with pT1 tumors had nodal disease, whereas 23 of 49 (47%) with pT2 or more advanced tumors had nodal disease. One patient who underwent a radical second resection died during the hospital stay for definitive resection, giving an in-hospital mortality rate of 2%.

Long-term outcomes after extended radical cholecystectomy

Of the 52 patients who underwent an extended radical cholecystectomy, 34 survived for more than 5 years and 18 died within 5 years. OS following extended radical cholecystectomy was 65% at 5 years and 53% at 10 years (median survival time, not reached) across the study cohort. OS was significantly worse in the 5 patients undergoing a noncurative (R1-2) resection than in the 47 patients undergoing a potentially curative (R0) resection ($P < 0.001$; Figure 3); the R1-2-resected patients died of recurrence within 5 years. This result indicated that R0 resection is a prerequisite for long-term survival after extended radical cholecystectomy.

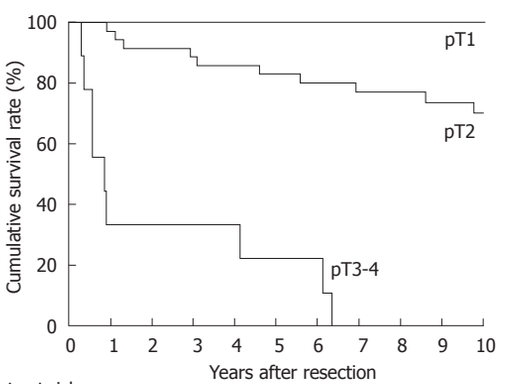
We then focused on the subgroup of 47 patients who had undergone an R0 resection for subsequent survival analyses. OS after R0 resection differed according to the pT classification ($P < 0.001$; Figure 4) and nodal status ($P = 0.010$; Figure 5). At the time of last follow-up, 11 patients with node-positive disease had survived for more than 5 years, suggesting that regional lymphadenectomy could achieve an acceptable rate of long-term survival for node-positive patients given an R0 resection (Figure 5).



No. of patients at risk

R0 resection	47	40	38	37	36	34	33	29	25	24	21
R1-2 resection	5	2	2	2	1	0	0	0	0	0	0

Figure 3 Kaplan-Meier overall survival estimates stratified for residual tumor status (R) across the study cohort (52 patients). The median overall survival (OS) was not reached with a 5-year OS rate of 72% in patients undergoing an R0 resection, whereas it was 11 mo with a 5-year OS rate of 0% in patients undergoing an R1-2 resection ($P < 0.001$).



No. of patients at risk

pT1	3	3	3	3	3	3	3	3	2	2	2
pT2	35	34	32	31	30	29	28	26	23	22	19
pT3-4	9	3	3	3	3	2	2	0	0	0	0

Figure 4 Kaplan-Meier overall survival estimates stratified for pathological T classification in 47 patients who underwent an no residual tumor resection. The median overall survival (OS) was not reached with a 5-year OS rate of 100% in patients with pathological T1 (pT1) disease. The median OS was not reached with a 5-year OS rate of 83% in pT2 patients, whereas it was 10 mo with a 5-year OS rate of 22% in pT3-4 patients. OS differed significantly among the groups ($P < 0.001$).

Five-year survival according to pT classification

All patients with pT1 tumor and most (29 of 36) patients with pT2 tumor survived for more than 5 years, while most (10 of 12) patients with pT3 tumor and the only patient with pT4 tumor died within 5 years (Table 3).

Of the 12 patients with pT3 tumors, 3 had an R1-2 resection, 2 had distant metastasis (pM1) in the liver, 2 had extensive extrahepatic organ involvement, and 1 had both an R1-2 resection and pM1 in the liver. All of these patients died within 5 years. Of the remaining four pT3 patients who had localized hepatic spread through the gallbladder fossa and underwent an R0 resection, 2 survived for more than 5 years, 1 survived for 4 years and 2 mo, and another succumbed to the cancer 4 mo after resection. Taken together, the above observations indicated that extended radical cholecystectomy was effective for most T1-2 lesions and some pT3 lesions with localized hepatic spread.

Table 3 Five-year survival according to the extent of tumor spread

Extent of tumor spread[1]	No. of 5-yr survivors (n = 34)	No. of non-5-yr survivors (n = 18)
pT1 (n = 3)		
pT1a pN0 pM0 (Stage Ⅰ)	2	0
pT1b pN0 pM0 (Stage Ⅰ)	1	0
pT2 (n = 36)		
pT2 pN0 pM0 (Stage Ⅱ)	20	1
pT2 pN1 pM0 (Stage ⅢB)	5	2
pT2 pN2 pM0 (Stage ⅣB)	4	3
pT2 pN2 pM1 (Stage ⅣB)	0	1
pT3 (n = 12)		
pT3 pN0 pM0 (Stage ⅢA)	0	3
pT3 pN1 pM0 (Stage ⅢB)	2	3
pT3 pN2 pM0 (Stage ⅣB)	0	1
pT3 pN0 pM1 (Stage ⅣB)	0	2
pT3 pN2 pM1 (Stage ⅣB)	0	1
pT4 (n = 1)		
pT4 pN2 pM0 (Stage ⅣB)	0	1

[1]According to the American Joint Committee on Cancer Cancer Staging Manual (7th edition)[19]. pT: Pathological T classification; pN: Pathological regional lymph nodes classification; pM: Pathological distant metastasis classification.

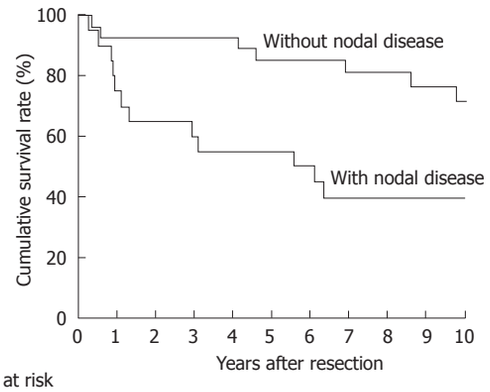
Table 4 Eleven 5-year survivors who underwent "extended" radical cholecystectomy for node-positive gallbladder cancer

Patient No.	Age (yr)	Sex	pT	Lymph node status	
				Location of positive nodes	No. of positive nodes
1	58	M	2	Hepatic artery	1
2	67	F	2	Cystic duct	1
3	68	F	2	Cystic duct	1
4	77	M	3	Cystic duct	1
5	75	F	3	Pericholedochal	1
6	62	F	2	Cystic duct	1
7	63	F	2	Cystic duct and posterosuperior pancreaticoduodenal	2
8	60	F	2	Cystic duct	2
9	67	F	2	Retroportal	2
10	55	F	2	Cystic duct and hepatic artery	2
11	57	F	2	Paraaortic	9[1]

[1]Isolated paraaortic nodal metastases. pT: Pathological T classification; M: Male; F: Female.

Five-year survival according to lymph node status

Among the eleven 5-year survivors with node-positive disease (Table 4), the number of positive lymph nodes was limited to one or two in 10 patients (patient No. 11 underwent an aggressive paraaortic lymphadenectomy for a total of nine positive paraaortic nodes), suggesting that extended radical cholecystectomy was effective in patients with up to a modest degree of regional nodal disease (up to two positive nodes).



No. of patients at risk

Without nodal disease	27	25	25	25	25	23	23	21	17	16	15
With nodal disease	20	15	13	12	11	11	10	8	8	8	6

Figure 5 Kaplan-Meier overall survival estimates stratified for nodal status in 47 patients who underwent an no residual tumor resection. The median overall survival (OS) was not reached with a 5-year OS rate of 85% in patients without nodal disease, whereas it was 67 mo with a 5-year OS rate of 55% in patients with nodal disease (P = 0.010).

Prognostic factors after extended radical cholecystectomy

Among the 15 conventional variables, age, pT classification, pN classification, pM classification, histological type, histological grade, lymphatic vessel invasion, venous invasion, perineural invasion, and residual tumor status were identified as significant prognostic factors on univariate analyses (Table 2). Timing of radical resection (initial resection *vs* second resection) did not affect OS rates after resection (P = 0.404).

The 10 univariately significant variables were then entered into multivariate analyses, with pT classification, pM classification, and histological type identified as independently significant variables (Table 2).

DISCUSSION

A standardized resection procedure has not been established for gallbladder cancer due to the diverse modes of spread possible with this disease[5,23]. Thus, the scope of radical resection when treating gallbladder cancer should be tailored based on the extent of spread in individual patients. Here, we analyzed 52 patients undergoing extended radical cholecystectomy and found it to be effective for pT1-2 tumors and some pT3 tumors with localized hepatic invasion, provided that the regional nodal disease was absent or only present to a modest degree (one or two positive lymph nodes).

In 1954, Glenn *et al*[3] first proposed a radical surgical procedure, designated as "radical cholecystectomy", where the gallbladder fossa and the node-bearing tissues within the hepatoduodenal ligament (portal lymph node dissection) were excised *en bloc* at the time of cholecystectomy. Extended radical cholecystectomy in our department (Figure 1) differs from the original Glenn and Hays procedure in the extent of lymphadenectomy and the presence or absence of bile duct resection. The extent of lymphadenectomy in the former procedure includes both the first- and the second-echelon node groups[11,15], but that in the original procedure was more limited[3,6,14]. The difference in the extent of lymphadenectomy may explain, in part, the acceptable rate of long-term survival (55%) achieved in our node-positive patients (Figure 5). Bile duct resection provides better local control by removing the possible presence of ductal (periductal) spread[21] as well as better clearance of nodal disease (especially periductal nodal disease). Thus, we believe that extended radical cholecystectomy confers oncological advantages over the original radical procedure.

The main modes of hepatic spread from resectable gallbladder cancer involve both direct invasion and portal tract invasion (lesions within the portal tracts of adjacent liver), the latter of which features intrahepatic lymphatic invasion[5,23-25]. We previously reported that the portal tract invasion is seen only in the vicinity (within about 1 cm) of the advancing margin of direct liver invasion[24,25]. Thus, a hepatectomy margin of approximately 2 cm or more in our extended radical cholecystectomy appears to be sufficient. The fact that local recurrence in hepatectomy margins did not occur in any of our patients (data not shown) also supports the validity of our 2 cm hepatectomy margin. When performing a wedge hepatectomy for invasive tumor, the entire cystic plate should be resected (Figure 2) because incomplete excision of the cystic plate violates the subserosal plane of the gallbladder and thus may leave behind tumor cells in this plane. Also, complete excision of the cystic plate facilitates removal of the adipose tissue within the triangle of Calot, which usually contains cystic duct node(s).

The current study showed that most patients with pT2 gallbladder cancer who underwent an extended radical cholecystectomy survived for more than 5 years (Figure 4), suggesting that pT2 tumors are the best candidates for this procedure. Regarding pT3-4 tumors, we cannot draw a definitive conclusion due to the small number of patients analyzed in this study. However, the fact that three of the four pT3 patients with localized invasion of the liver through the gallbladder fossa survived for more than 4 years suggests that such pT3 patients may benefit from extended radical cholecystectomy, provided that they have no distant metastases and undergo an R0 resection. Our results also suggest that a considerable proportion of node-positive patients may benefit from this procedure (Figure 5). By analyzing 5-year survivors with nodal disease (Table 4), we realized that this procedure might clear only a modest degree of nodal disease (one or two positive nodes in our cohort). Taken together, the above observations indicate that the extended radical cholecystectomy undertaken in our department provides survival benefit for most patients with pT2 tumors and some patients with pT3 tumors with localized hepatic invasion, provided that the nodal disease is limited to a modest degree and an R0 resection is feasible.

In 2007, Yokomizo *et al*[26] argued that hepatectomy and/or bile duct resection could be withheld in radical surgery for pT2 gallbladder cancer provided that negative resection margins are achieved. The current NCCN guidelines[6] recommend hepatectomy and lymphadenectomy

for pT1b or greater tumors, whereas bile duct resection is suggested as optional. We previously reported that a considerable proportion (40%) of pT2 patients could survive for more than 5 years after cholecystectomy alone if the resection margins are negative[12]. The above observations indicate that bile duct resection, hepatectomy, and even lymphadenectomy could be omitted for some pT2 patients. Indeed, some of our pT2 patients with advanced age or comorbid disease(s) had a less aggressive resection by omitting bile duct resection and/or hepatectomy (Table 1). However, considering the excellent outcomes after extended radical cholecystectomy for pT2 tumors (Figure 4), we now usually recommend application of the extended radical cholecystectomy to robust patients with pT2 gallbladder cancer.

The authors' policy on radical surgery for gallbladder cancer is to select a resection procedure for each patient based on the extent of tumor spread in the patient. Although extended radical cholecystectomy has been considered the standard procedure for fairly localized tumors in our department, only one-third (52 of 145) of our patients underwent this procedure (Table 1). The current study implies that extensive pT3 disease, pT4 disease, or marked nodal disease is beyond the scope of extended radical cholecystectomy. Such tumors may require more extensive resections (major hepatectomy, pancreaticoduodenectomy, *etc*.) for clearance of the locoregional disease[11]. Adequate selection of a resection procedure in individual patients is mandatory to improve survival for gallbladder cancer.

The main limitations of the current study include the retrospective nature of the analysis and the small number of patients spanning a long period of time. We believe, however, that these limitations did not greatly affect the results of the study as the differences between the groups were too marked to have resulted from bias. In addition, the role of extended radical cholecystectomy in surgical management strategy for gallbladder cancer is now more clearly defined than previously[11,12,16]. Our results thus provide useful information for selecting an adequate resectional procedure for individual patients with gallbladder cancer.

In conclusion, extended radical cholecystectomy is indicated for gallbladder cancer patients with pT2 tumors and for some with pT3 tumors with localized hepatic invasion, provided that the regional nodal disease is limited to a modest degree (up to two positive nodes). Extensive pT3 disease, pT4 disease, or marked nodal disease appears to be beyond the scope of this procedure and thus may require more extensive resection. This study confirms that extended radical cholecystectomy plays a key role in the surgical management of invasive gallbladder cancer if the spread is moderate.

COMMENTS

Background

In 1954, Glenn and Hays first proposed "radical cholecystectomy", which has been used worldwide for localized gallbladder cancer. "Extended" radical cholecystectomy (designated as the modified Glenn operation) was instituted in the study department in 1982. Although the authors' procedure has been widely used among Japanese surgeons, indications for this particular procedure have not yet been established.

Research frontiers

As a standardized resection procedure has not been defined for gallbladder cancer, the scope of radical resection should be tailored based on the extent of spread in individual patients. However, adequate selection of a resection procedure in individual patients remains yet to be established.

Innovations and breakthroughs

The current study clearly defines the role of extended radical cholecystectomy in surgical management strategy for gallbladder cancer. Briefly, **extended radical cholecystectomy is indicated for pathological T2 (pT2) tumors and for some pT3 tumors with localized hepatic invasion, provided that the nodal disease is limited to a modest degree (up to two positive nodes). Extensive pT3 disease, pT4 disease, or marked nodal disease appears to be beyond the scope of this procedure and thus may require more extensive resection.**

Applications

The current study provides practical information for selecting an adequate resectional procedure for individual patients with gallbladder cancer.

Terminology

Radical cholecystectomy (Glenn operation): *en bloc* excision of the gallbladder, gallbladder fossa, and the node-bearing tissues within the hepatoduodenal ligament; **"Extended" radical cholecystectomy (modified Glenn operation):** *en bloc* excision of the gallbladder, gallbladder fossa, extrahepatic bile duct, and the regional lymph nodes (first- and second-echelon node groups).

Peer review

Overall, this paper is well written, concise and informative. "Extended" radical cholecystectomy involves more extensive regional lymphadenectomy compared with the "original" radical cholecystectomy proposed by Glenn and Hays. This has led to achievement of good long-term survival in node-positive patients.

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BRIEF ARTICLE

Clinicopathologic significance of expression of nuclear factor-κB RelA and its target gene products in gastric cancer patients

Hyuk-Chan Kwon, Sung-Hyun Kim, Sung Yong Oh, Suee Lee, Ji Hyun Lee, Jin Seok Jang, Min Chan Kim, Ki Han Kim, Su-Jin Kim, Seong-Geun Kim, Hyo-Jin Kim

Hyuk-Chan Kwon, Sung-Hyun Kim, Sung Yong Oh, Suee Lee, Ji Hyun Lee, Jin Seok Jang, Hyo-Jin Kim, Department of Internal Medicine, Dong-A University College of Medicine, Busan 602-715, South Korea
Min Chan Kim, Ki Han Kim, Department of Surgery, Dong-A University College of Medicine, Busan 602-715, South Korea
Su-Jin Kim, Department of Pathology, Dong-A University College of Medicine, Busan 602-715, South Korea
Seong-Geun Kim, Research Institute for Convergence of Biomedical Science and Technology, Pusan National University Yangsan Hospital, Busan 602-715, South Korea
Author contributions: Kim SJ and Kim HJ designed research; Kim MC and Kim KH contributed to analytic tools; Lee S, Lee JH and Jang JS analyzed data; and Kim SG, Kwon HC, Kim SH and Oh SY wrote the paper.
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Correspondence to: Hyo-Jin Kim, MD, PhD, Department of Internal Medicine, Dong-A University College of Medicine, 3-1 Dongdaeshin-dong, Seo-gu, Busan 602-715, South Korea. kimhj@dau.ac.kr
Telephone: +82-51-2402951 Fax: +82-51-2402088
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Abstract

AIM: To assess the prognostic significance of nuclear factor-κB (NF-κB) and its target genes in gastric cancer.

METHODS: The tumor tissues of 115 patients with gastric cancer were immunohistochemically evaluated using monoclonal antibodies against NF-κB RelA. Preoperative serum levels of vascular endothelial growth factor (VEGF), interleukin-6 (IL-6) were assessed *via* enzyme-linked immuno-sorbent assay. C-reactive protein (CRP) and serum amyloid A (SAA) were measured *via* immunotrubidimetry.

RESULTS: Positive rate of NF-κB RelA was 42.6%. NF-κB RelA expression in tumor tissues was also related to serum levels of IL-6 (P = 0.044) and CRP (P = 0.010). IL-6, SAA, CRP were related to depth of invasion, VEGF and SAA were correlated with lymph node metastasis. IL-6, VEGF, SAA and CRP were related to the stage. Univariate analysis demonstrated that immunostaining of NF-κB RelA, levels of IL-6, VEGF, SAA were significantly related with both disease free survival and overall survival (OS). Multivariate analysis verified that NF-κB RelA [hazard ratio (HR): 3.40, P = 0.024] and SAA (HR: 3.39, P = 0.045) were independently associated with OS.

CONCLUSION: Increased expression of NF-κB RelA and high levels of serum SAA were associated with poor OS in gastric cancer patients.

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Key words: Nuclear factor-κB; Vascular endothelial growth factor; Interleukin-6; C-reactive protein; Serum amyloid A; Stomach; Carcinoma

Peer reviewer: Dr. Fei Chen, Department of Pharmaceutical Sciences, Wayne State University, 259 Mack Avenue, Detroit, MI 48201, United States

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INTRODUCTION

Gastric cancer continues to pose a significant health prob-

lem, despite its declining incidence in the West. It is the 4th most common cancer worldwide, accounting for 8.6% of all new cancer diagnoses in 2002[1]. In South Korea, gastric cancer is the most common carcinoma in men, and the third-most common cancer type in women[2]. Surgery is the only potentially curative treatment for localized gastric cancer. However, the majority of patients will relapse after definitive surgery, and the 5-year overall survival (OS) rates of this condition remain lower than 30% to 40% in Western countries[1]. These high levels of gastric cancer mortality can be attributed to the high incidence of serosal invasion, direct invasion into the adjacent organs, peritoneal seeding, lymph node metastasis, and distant metastasis of gastric cancer. The assessment of biological prognostic factors is of great clinical importance in gastric cancer. The widely used tumor node metastasis (TNM) staging exhibits high prognostic power, but cannot predict outcomes perfectly for particular individuals. The outcome of cancer patients may be affected by variability in tumor biology. Thus, tumors with similar clinical or pathologic characteristics frequently evidence different clinical outcomes. Gaining insight into the underlying molecular mechanisms of initiation and progression of gastric cancer will be a necessary step in definitively identifying groups of patients with poor prognoses. The transcription factor nuclear factor-κB (NF-κB) is a heterodimeric complex of the Rel-family proteins. The most common dimer is the RelA (p65)/p50 heterodimer, i.e., NF-κB[3]. NF-κB is sequestered in the cytoplasms in an inactive form *via* interaction with the inhibitor of nuclear factor-κB proteins (IκBs). A variety of stimuli, including cytokines and infectious agents, induce the phosphorylation, ubiquitination, and degradation of IκBs, and also allow the nuclear translocation of NF-κB[4]. Once in the nucleus, NF-κB binds to DNA at its response elements and activates the expression of genes. Recent studies using anti-RelA immunostaining have shown that NF-κB is constitutively activated in gastric cancer[5-7], Additionally, there is some evidence to suggest that NF-κB levels are higher in gastric cancer cells than in normal adjacent epithelial cells, and that NF-κB activation in gastric cancer is associated with lymphatic invasion, advanced stage, and poor clinical outcomes[5,7]. However, other investigators have reported conflicting data with regard to the relationship of NF-κB protein with patient survival[6].

The active NF-κB transcription factor promotes the expression of more than 150 target genes[8]. The majority of proteins encoded for by NF-κB target genes participate in host immune responses, whereas others appear to be involved in the proliferation, invasion, angiogenesis, and metastasis of cancer cells. NF-κB promotes the production of cytokines that function as growth factors for transformed enterocytes[9]. One of these growth factors was subsequently identified as interleukin-6 (IL-6), which is encoded for by a NF-κB target gene. IL-6 acts on a broad range of tissues and cell lines, and induces cell growth and differentiation, the production and expression of other cytokines, and acute-phase protein synthesis. IL-6 also promotes growth arrest[10], and promotes angiogenesis *via* the induction of vascular endothelial growth factor (VEGF) expression[11]. A previous study reported that the preoperative serum level of IL-6 was associated with lymph node metastasis, depth of invasion, advanced stage, and poor survival in gastric cancer patients[12].

C-reactive protein (CRP) and serum amyloid A (SAA) are nonspecific, acute-phase, hepatic proteins secreted in response to cytokines, including IL-1, IL-6, and tumor necrosis factor-α[13]. CRP is a representative marker for inflammatory conditions, and carries out a crucial anti-infection function in the immune system. In many cancers, chronic inflammation has reportedly been involved with malignant change, and the risks of colorectal cancer appear to be increased when pre-diagnostic CRP levels are high[14]. Cancer invasion begins with inflammation around cancer cells. Thus, serum CRP levels have generally been shown to be higher in cases of invasive cancer than in cases of non-invasive cancer[12].

An increasing body of *in vitro* evidence supports the involvement of SAA in carcinogenesis and neoplastic diseases[15]. An accumulating body of evidence has been collected to suggest that SAA might be included in a group of biomarkers for the detection of a pattern of physiological events that reflects the growth of malignancy and host response. There have also been some reports indicating that the SAA-induced upregulation of matrix metalloproteinase 9 was mediated *via* formyl peptide receptor like-1 and was achieved at the transcriptional level *via* NF-κB[16], and also that preoperative SAA was useful in predicting the survival of gastric cancer patients[17].

The expression of several angiogenic factors is also known to be regulated by NF-κB. Macrophages as well as tumor cells have been shown to generate VEGF under the control of NF-κB activation[18]. VEGF is the most important pro-angiogenic growth factor, and its stimulation under hypoxic conditions carries out critical functions in promoting the survival of malignant cells, in local tumor growth and invasion, and in the development of metastases[19]. We demonstrated that VEGF expression was correlated with the depth of invasion and NF-κB expression in stage Ⅲ colorectal cancer patients[20].

The objective of the current investigation was to evaluate the prognostic significance of NF-κB RelA and its target gene products in gastric cancer patients.

MATERIALS AND METHODS

Patients

From March 2005 to July 2006, a total of 115 patients with histologically-confirmed gastric carcinomas at the Dong-A University Hospital were enrolled in this study. After operation, the disease status of patients was evaluated *via* gastroendoscopy and abdominal computed tomography (CT). During the first 2 years, we checked CT and endoscopy at 6-mo intervals, and afterwards CT and endoscopy were conducted every 12 mo. We also carried out abdomen CT when we observed abdominal distension, acute abdominal pain, severe diarrhea and

chest X-ray abnormalities suggestive of lung metastasis. Endoscopy was conducted in cases in which patients complained of unexplained abdominal discomfort and dyspepsia. On average, in the stage Ⅲ and Ⅳ patients, the first evaluation was conducted within 3 mo after operation. All patients provided informed consent, and the hospital review board approved the study.

Construction of tissue microarray

Two cores of 2-millimeters in diameter of formalin-fixed, paraffin-embedded tissue blocks were obtained from each gastric cancer patient's sample and arranged in new recipient paraffin blocks using an UNITMA apparatus (UNITMA, Seoul, South Korea) after careful histological assessment. All cases except for one endoscopic biopsy sample and the normal control tissue, were embedded in 6-tissue microarray blocks. Tissue microarray blocks were serially cut into 4 μm sections and stained for routine hematoxylin and eosin staining and immunohistochemistry.

Immunohistochemistry

All immunohistochemistry processes were automatically performed using a Ventana autostainer (Benchmark; Ventana Medical Systems, Tuscon, Arizona, United States). For antigen retrieval, retrieval solution (Ventana) was automatically poured onto the sections, and heated for 60 min at 100 ℃. Endogenous peroxide activity was blocked *via* immersion in 3% hydrogen peroxide for 4 min. With diluted primary antibody for NF-κB p65 (rabbit monoclonal antibody, 1:250, Epitomics, CA, United States), the tissue sections were incubated for 32 min at 36 ℃. Immunoperoxidase staining was carried out using a DAB system (iView DAB detection kit, Ventana) and the sections were counterstained lightly with hematoxylin.

Interpretation of immunohistochemical staining

The percentage and intensity of the immunoreactive tumor cells in each core was recorded and the final values of the positive tumor cells were evaluated as the mean of the immunoreactivity in three cores. The presence of tumor tissue in at least two interpretable cores was required for the inclusion of a case in the statistical analysis. All of the slides were evaluated independently by an experienced pathologist (Kim SJ) who was blinded to all of the clinicopathologic data. Immunostaining for NF-κB RelA was regarded as positive when tumor cells evidenced granular cytoplasmic immunostaining of the lesion, with a minimal background. The percentage scoring of the immunoreactive tumor cells was as follows: 0 (0%), 1 (1%-10%), 2 (11%-50%) and 3 (> 50%). The staining intensity was visually scored and stratified as follows: 0 (negative), 1 (weak, if it was a blush), and 2 (strong, if it was obviously positive at × 20 magnification). A final score was obtained for each case by multiplying the percentage by the intensity score. Therefore, tumors with multiplied scores exceeding 4 (i.e., tumors with a strong intensity of > 10% of the tumor cells) were regarded as positively immunoreactive to NF-κB RelA; all other scores were considered negative.

Assay of serum IL-6, VEGF, SAA and CRP

Peripheral venous blood samples were obtained within 7 d prior to gastrectomy, collected in plain tubes, permitted to clot, and centrifuged for 10 min at 3000 r/min at 4 ℃ within one hour of collection to obtain the serum. The sera were aliquoted and stored at -80 ℃ until being assayed. The VEGF and IL-6 levels from the sera of patients and the standard solutions supplied were measured using a commercial system (Quantikine hVEGF, hIL-6 Immunoassay, R and D Systems, United States) in accordance with the manufacturer's instructions. All serum samples were measured by an investigator who was blinded to the clinical data. The blood samples for CRP analysis were collected in serum separation tubes, and the serum CRP levels were measured *via* immunoturbidimetry (Denka Seiken Co. Ltd., Japan). SAA protein was measured *via* latex agglutination turbidoimmunometric assays (Eiken Chemical Co., Japan).

Statistical analysis

The associations between NF-κB RelA expression and the relevant clinicopathological parameters were assessed *via* an χ^2-test or Fisher's exact test. Serum levels of VEGF, IL-6 and CRP were expressed as mean values. The Mann-Whitney *U*-test was used to evaluate the relationship between protein concentration and clinicopathological features. Disease-free survival (DFS) was defined as the length of time from surgery to initial recurrence of disease. OS was defined as the length of time from surgery to death. The Kaplan-Meier method was utilized to construct curves for DFS and OS. Data on patients who died without any evidence of disease recurrence were censored at the time of death for DFS calculations. The log-rank test was employed to compare distributions. In order to identify the independent factors that were significantly related to patient prognosis, we utilized Cox's proportional hazard analysis *via* a stepwise procedure. All tests were two-sided, and $P < 0.05$ was considered statistically significant. Analyses were conducted using SPSS version 18.0 (SPSS, Chicago, IL, United States).

RESULTS

Patient characteristics

The mean age of the patients was 59 years (range: 24-75 years), and the study population included 47 (40.92%) females and 68 (59.1%) males. Thirty-six of the patients (31.3%) underwent radical total gastrectomy, 75 (65.2%) underwent radical subtotal gastrectomy, and four patients (3.5%) did not have operation due to distant metastasis. The majority of the tumors were located in the middle or lower stomach (72.1%), and were moderate to poorly differentiated carcinomas (75.7%). Thirty-four of the patients (30.6%) had pathological T3 (pT3) or pT4 tumors, 69 (63.2%) had lymph node metastases, and 26 patients (23.4%) had a positive lymph node ratio of ⩾ 0.2. The postoperative stages of the patients were Ⅰ, Ⅱ, Ⅲ and Ⅳ in 43 (37.4%), 23 (20.0%), 31 (27.0%) and 18 (15.7%)

Table 1 Correlation of nuclear factor-κB RelA expression and clinicopathological findings *n* (%)

	NF-κB RelA (-)	NF-κB RelA (+)	*P*
Sex			1.000
Male	39 (57.4)	29 (42.6)	
Female	27 (57.4)	20 (42.6)	
Age (yr)			0.192
< 60	38 (63.3)	22 (36.7)	
⩾ 60	28 (50.9)	27 (49.1)	
Site			0.002
Upper	9 (37.5)	15 (62.5)	
Middle	9 (50.0)	9 (50.0)	
Lower	48 (72.7)	18 (27.3)	
Diffuse	0 (0)	3 (100)	
Size (cm)			0.045
< 5	47 (67.1)	23 (32.9)	
⩾ 5	19 (46.3)	22 (53.7)	
Lauren			0.988
Intestinal	27 (58.7)	19 (41.3)	
Diffuse	26 (57.8)	19 (42.2)	
Mixed	9 (60.0)	6 (40.0)	
Differentiation			0.631
Well	11 (68.8)	5 (31.3)	
Moderate	14 (50.0)	14 (50.0)	
Poor	34 (60.7)	22 (39.3)	
Mucinous	7 (63.6)	4 (36.4)	
Lymphovascular invasion			0.012
-	44 (69.8)	19 (30.2)	
+	22 (45.8)	26 (54.2)	
T stage			0.012
1-2	52 (67.5)	25 (32.5)	
3-4	14 (41.2)	20 (58.8)	
Node			< 0.001
-	34 (81.0)	8 (19.0)	
+	32 (46.4)	37 (53.6)	
Lymph node ratio			< 0.001
< 0.2	60 (70.6)	25 (29.4)	
⩾ 0.2	6 (23.1)	20 (76.9)	
Stage			< 0.001
1	36 (83.7)	7 (16.3)	
2	14 (60.9)	9 (39.1)	
3	16 (51.6)	15 (48.4)	
4	0 (0)	18 (100)	
CEA (ng/mL)			0.134
< 5	64 (59.3)	44 (40.7)	
⩾ 5	2 (28.6)	5 (71.4)	
CA19-9 (U/mL)			< 0.001
< 37	63 (67.7)	30 (32.3)	
⩾ 37	3 (13.6)	19 (86.4)	

NF-κB: Nuclear factor-κB; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9.

patients, respectively. With regard to the Lauren classifications, 46 (40.0%) patients were of the intestinal type, 43 (37.4%) of the diffuse type, and 15 (13.0%) of the mixed type. Sixteen patients (14.4%) had well, 28 (25.2%) had moderate, and 56 (50.5%) had poorly differentiated adenocarcinoma. Fifty-five patients (47.8%) received oral 5-fluorouracil, and 46 patients (40.0%) received cisplatin-based adjuvant chemotherapy after gastrectomy.

Correlations between the NF-κB RelA expression, target gene expression and clinicopathological parameters

A representative tumor specimen evidencing increased NF-κB RelA expression in the cytoplasm is shown in Figure 1. NF-κB RelA expression was found to be significantly correlated with several findings (Table 1). NF-κB RelA expression was more common in cases of more than 5 cm ($P = 0.045$), deeper invasion ($P = 0.012$), lymph node metastases ($P < 0.001$), advanced stage ($P < 0.001$), positive lymphovascular invasion ($P = 0.012$), and increased Carbohydrate antigen 19-9 ($P < 0.001$). However, no significant correlations were noted between Nf-κB a RelA nd other parameters. We noted that VEGF, SAA levels were significantly correlated with tumor size ($P = 0.022$, $P < 0.001$, respectively). IL-6, SAA and CRP levels were related to the depth of invasion ($P = 0.007$, $P < 0.001$, $P = 0.015$, respectively). We also noted significant differences in serum VEGF and SAA levels between patients with lymph node metastasis and those without lymph node metastasis ($P < 0.001$, respectively). The mean levels of IL-6, VEGF, SAA and CRP were increased proportionally with the cancer stage ($P = 0.031$, $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively). Additionally, NF-κB RelA expression was correlated positively with IL-6 ($P = 0.044$), and CRP ($P = 0.010$) levels (Table 2).

Table 2 Correlation of nuclear factor-κB RelA and level of target genes (mean ± SD)

	Nuclear factor-κB		*P*
	- (*n* = 66)	+ (*n* = 49)	
IL-6 (pg/mL)	8.1 ± 8.6	11.8 ± 10.7	0.044
VEGF (pg/mL)	117.2 ± 154.2	178.9 ± 202.7	0.066
SAA (mg/mL)	8.1 ± 20.3	14.5 ± 16.3	0.072
CRP (mg/mL)	0.1 ± 0.1	0.3 ± 0.4	0.010

IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor; SAA: Serum amyloid A; CRP: C reactive protein.

NF-κB RelA expression, target gene expressions and clinical outcomes

The median follow-up duration of the patients was 66.6 mo (range: 9.3-88.8 mo). Univariate analysis of clinicopathologic parameters and DFS and OS are provided in Table 3. Tumor size, location, lymphovascular invasion, stage, and positive lymph node ratio were significantly associated with both 5-year DFS and OS. Positive correlations were detected between immunostaining of NF-κB RelA, levels of IL-6, VEGF, SAA, and DFS and OS. CRP levels were not correlated with 5-year DFS or OS. The OS curves of SAA and NF-κB RelA are provided in Figure 2. In order to determine the independent prognostic values of these markers, we employed a multivariate Cox proportional hazard analysis to control for other prognostic factors (Table 4). Accordingly, stage was identified as an independent prognostic factor of both DFS [hazard ratio (HR): 7.83, 95% confidence interval (CI) = 2.44-25.18, $P = 0.001$], and OS (HR: 6.15, 95% CI = 1.97-19.22, $P = 0.002$). Positive lymph node ratio was also identified as a significant predictor of both DFS (HR: 1.68, 95% CI = 1.01-2.79, $P = 0.043$), and OS (HR: 2.46, 95% CI = 1.01-5.94, $P = 0.047$), after controlling for other clinicopathologic parameters. The expressions of NK-κB and

Table 3 Univariate analysis according to the clinicopathologic finding

	n	5-yr DFS (%)	*P*	5-yr OS (%)	*P*
Sex					0.824
Male	68	64.7	0.827	65.8	
Female	47	68.1		70.2	
Age (yr)					0.367
< 60	60	70	0.355	71.7	
⩾ 60	65	61.8		62.7	
Site					< 0.001
Upper	24	41.7	< 0.001	45.8	
Middle	18	72.2		77.8	
Lower	16	80.3		79.8	
Diffuse	3	0		0	
Size (cm)					0.005
< 5	70	77.1	0.006	78.1	
⩾ 5	41	53.7		56.1	
Lauren					0.301
Intestinal	46	71.7	0.345	73.3	
Diffuse	43	60.5		62.8	
Mixed	15	80		80.8	
Differentiation					0.779
Well	16	68.8	0.879	75	
Moderate	28	75.2		75	
Poor	56	66.1		67	
Mucinous	11	63.6		63.6	
Lymphovascular invasion					< 0.001
-	63	82.5	< 0.001	82	
+	48	50		54.2	
T stage					< 0.001
1-2	77	79.2	< 0.001	80	
3-4	34	44.1		47.1	
Node					< 0.001
-	42	97.6	< 0.001	97.6	
+	69	50.7		52.9	
Lymph node ratio					< 0.001
< 0.2	85	81.2	< 0.001	81	
⩾ 0.2	26	26.9		26.9	
Stage					<0.001
1	43	100	< 0.001	100	
2	23	60.9		63.2	
3	31	54.8		58.1	
4	18	11.1		11.1	
CEA (ng/mL)					< 0.001
< 5	108	69.4	< 0.001	69.4	
⩾ 5	7	14.3		14.3	
CA19-9 (U/mL)					< 0.001
< 37	93	73.1	< 0.001	73	
⩾ 37	22	36.4		36.4	
Nuclear factor-κB					< 0.001
-	66	90.9	< 0.001	90.8	
+	49	32.7		32.7	
IL-6 (pg/mL)					< 0.001
< 6.83	59	81.4	< 0.001	81.4	
⩾ 6.83	56	50		49.6	
VEGF (pg/mL)					0.002
< 68.1	57	80.7	0.001	80.5	
⩾ 68.1	58	52.6		52.6	
SAA (mg/mL)					< 0.001
< 4.2	58	86.2	< 0.001	86	
⩾ 4.2	57	45.6		45.6	
CRP (mg/mL)					0.899
< 0.1	59	66.1	0.858	66.1	
⩾ 0.1	56	66.1		65.9	

DFS: Disease free survival; OS: Overall survival; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor; SAA: Serum amyloid A; CRP: C-reactive protein.

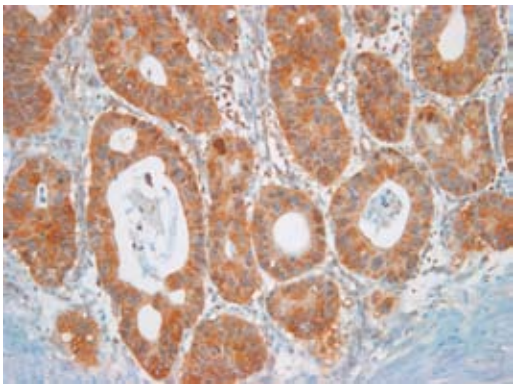


Figure 1 Immunohistochemical staining for nuclear factor-κB RelA in gastric cancer. Gastric cancer cells evidence intense cytoplasmic immunoreactivity for nuclear factor-κB (× 200; inset, × 40).

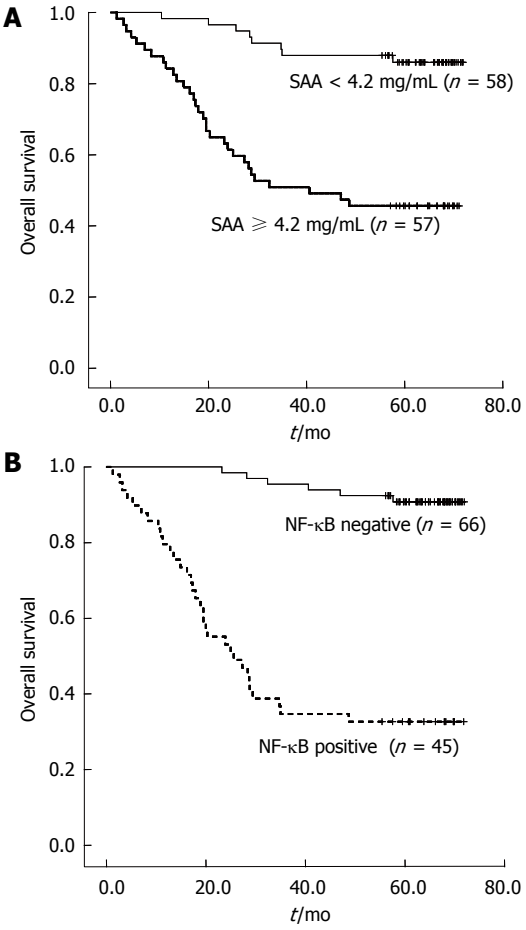


Figure 2 Overall survival curve. A: Serum amyloid A (SAA) expression ($P < 0.001$); B: Nuclear factor-κB (NF-κB) expression ($P < 0.001$).

SAA levels were shown to significantly affect OS on multivariate analysis (HR: 3.40, 95% CI = 1.17-9.87, $P = 0.024$; HR: 3.39, 95% CI = 1.02-11.23, $P = 0.045$, respectively).

DISCUSSION

There have been a few reports regarding the expression of NF-κB in human gastric cancer assessed by immu-

Table 4 Multivariate analysisclinicopathological findings

	5-yr disease free survival			5-yr overall survival		
	HR	95% CI	*P*	HR	95% CI	*P*
Stage	7.83	2.44-25.18	0.001	6.15	1.97-19.2	0.002
Site	1.21	0.66-2.22	0.536	1.22	0.67-2.23	0.510
Size	0.48	0.188-1.27	0.142	0.577	0.22-1.41	0.218
LN ratio	1.68	1.01-2.79	0.043	2.46	1.01-5.94	0.047
LVI	0.79	0.30-2.11	0.650	1.02	0.41-2.52	0.951
IL-6	1.40	0.53-3.66	0.488	1.19	0.45-3.14	0.726
VEGF	1.22	0.77-1.95	0.390	2.11	0.86-5.17	0.102
SAA	3.21	0.96-10.79	0.058	3.39	1.02-11.23	0.045
CEA	2.17	0.66-7.07	0.198	2.38	0.76-7.48	0.135
CA19-9	0.93	0.35-2.45	0.898	1.96	0.76-5.02	0.158
NF-κB RelA	2.87	0.96-8.49	0.057	3.40	1.17-9.87	0.024

LVI: Lymphovascular invasion; IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor; SAA: Serum amyloid A; LN: Lymph node; CEA: Carcinoembryonic antigen; NF-κB: Nuclear factor-κB; CA19-9: Carbohydrate antigen 19-9; HR: Hazard ratio; 95% CI: 95% confidence interval.

nohistochemistry using the same anti-RelA (p65) antibody[5,7]. The present study confirms constitutively active NF-κB RelA in gastric carcinoma, which is consistent with the findings of previous studies[5,7]. The expression rate of NF-κB RelA in gastric carcinoma is higher than that observed in normal adjacent epithelial cells, and NF-κB RelA expression was observed primarily in gastric carcinoma cytoplasm with an expression rate of 42.6%; this is somewhat higher than in other studies[5,7]. We showed that NF-κB RelA expression was correlated with tumor size, depth of invasion, lymph node metastasis, lymphovascular invasion and advanced stage. Furthermore, positive NF-κB RelA staining was associated with both DFS and OS, and NF-κB RelA was identified as an independent prognostic factor in multivariate analysis (HR: 3.40, 95% CI = 1.17-9.87, $P = 0.024$).

Sasaki *et al*[5] also reported that NF-κB activation was correlated with tumor size and lymphatic invasion. However, Lee *et al*[6] previously demonstrated that NF-κB activation was not correlated with poor patient prognosis, but rather was significantly associated with better prognosis of early stage gastric carcinoma patients. The absence of clear-cut criteria in the evaluation of NF-κB immunoexpression may be the major reason for the conflicts among the previously published data regarding the prognostic impact of this molecule in gastric cancer.

The activation of NF-κB regulates a variety of genes involved in the proliferation, invasion, angiogenesis, and metastasis of cancer cells[8]. In this study, NF-κB RelA expression was correlated positively with IL-6 and CRP levels. According to some studies attempting to evaluate the clinical significance of IL-6 in gastric cancer patients, IL-6 levels were higher in malignancies than in non-malignancies, increased with increasing tumor size and depth, and were associated with poor clinical outcomes[12,21]. We found significant correlations between elevated IL-6 levels and several conditions: large tumor size, deeper invasion depth, advanced stage, and poor DFS and OS.

Findings from studies of gastrointestinal cancer patients have demonstrated that those subjects with elevated serum CRP concentrations tended toward poorer prognoses than those whose CRP levels were not increases[14,22]. CRP was related to the depth of tumor invasion, tumor differentiation, and TNM stage. However, we found no significant associations between CRP and DFS or OS.

SAA is the most sensitive plasma protein indicative of inflammatory activity[23]. SAA is superior to CRP for the detection of inflammation associated with severe burns, viral infections, kidney transplantation, Crohn's disease, and ulcerative colitis[23]. In this study, SAA was related to tumor size, depth of invasion, lymph node metastasis, clinical stage, and cancer recurrence. Similar results have been reported in other studies[17]. Those studies showed that gastric cancer patients had higher SAA levels than did patients with gastric ulcers and healthy individuals, and that SAA concentrations were correlated with prognosis[17]. SAA was related significantly to DFS and OS, and was determined to be an independent prognostic factor for OS in multivariate analysis (HR: 3.39, 95% CI = 1.02-11.23, $P = 0.045$).

Tumor VEGF expression was identified as a significant marker for tumor recurrence or reduced survival independent of conventional clinicopathological variables in gastric cancer[24,25]. However, the prognostic value of VEGF expression in gastric cancer remains unclear, and some studies have indicated that tumor VEGF expression may constitute an independent prognostic factor in DFS[24,25], whereas others have reported no such association[26]. In our study, VEGF expression was significantly correlated with tumor size, lymph node invasion and staging; however, our findings do not appear to confirm a significant association between VEGF expression and unfavorable prognosis in multivariate analysis.

Univariate analysis demonstrated that the immunostaining of NF-κB RelA and levels of IL-6, VEGF and SAA were all significantly related with both DFS and OS. Multivariate analysis results verified that NF-κB RelA and SAA were independently associated with OS. Although changes in the expression levels of several cytokines have been frequently reported, their proposed role in the pathogenesis and progression of gastric cancer remains controversial. The results of such studies may be confounded by several factors, including small patient sample size and variable therapeutic protocols which may potentially have affected patient outcomes. Well-designed large scale studies will be necessary to confirm our data.

COMMENTS

Background

The active nuclear factor-κB (NF-κB) transcription factor promotes the expression of target genes. The majority of proteins encoded for by NF-κB target genes participate in host immune responses, proliferation, invasion, angiogenesis, and metastasis of cancer cells.

Research frontiers

The objective of the current investigation was to clarify the prognostic significance of NF-κB RelA and its target gene products in gastric cancer patients.

Innovations and breakthroughs

In the present study, NF-κB RelA expression in tumor tissues was related to increased serum levels of interleukin (IL)-6 (P = 0.044) and C reactive protein (CRP) (P = 0.010). Levels of serum IL-6, vascular endothelial growth factor (VEGF), serum amyloid A (SAA) and CRP were related to the stage. Univariate analysis demonstrated that immunostaining of NF-κB RelA, levels of IL-6, VEGF, SAA were significantly related with both disease free survival and overall survival (OS). Multivariate analysis verified that NF-κB RelA and SAA were independently associated with OS.

Applications

Increased expression of NF-κB RelA and high levels of serum SAA were associated with poor OS in gastric cancer patients.

Peer review

The authors of this manuscript examined potential prognostic value of NF-κB RelA expression in gastric cancer patients and reported an inverse relationship between RelA expression and patient survival. In addition to RelA, the contribution of several inflammatory mediators/cytokines to the patient prognosis was also investigated. The study was well-designed and the data are convincing.

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BRIEF ARTICLE

Prognosis of hepatocellular carcinoma expressing cytokeratin 19: Comparison with other liver cancers

Jung Il Lee, Jin-Woo Lee, Joon Mee Kim, Ja Kyung Kim, Hyun Jung Chung, Young Soo Kim

Jung Il Lee, Jin-Woo Lee, Young Soo Kim, Department of Internal Medicine, School of Medicine, Inha University, Incheon 400-711, South Korea
Joon Mee Kim, Department of Pathology, School of Medicine, Inha University, Incheon 400-711, South Korea
Ja Kyung Kim, Hyun Jung Chung, Department of Internal Medicine, Yonsei University College of Medicine, Gangnam Severance Hospital, Seoul 135-720, South Korea
Author contributions: Lee JI and Lee JW designed the study; Lee JI, Kim JM, Kim JK and Chung HJ performed the study; and Kim YS and Lee JI made the analyses and wrote the article.
Correspondence to: Jung Il Lee, MD, PhD, Associate Professor of Internal Medicine, Division of Gastroenterology and Hepatology, School of Medicine, Inha University, 7-206 Shinheung-Dong, Jung-Gu, Incheon 400-711, South Korea. mdflorence@inha.ac.kr
Telephone: +82-32-8902548 Fax: +82-32-8902549
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Abstract

AIM: To investigate whether expressing biliary phenotype predicted poor outcome after the surgical treatment in primary liver cancers.

METHODS: Out of 204 patients that underwent liver resection due to hepatocellular carcinoma (HCC), liver specimens of 70 patients with HCC were evaluated for biliary components by cytokeratin (CK) 19 immunostain ($CK19^-$ HCC and $CK19^+$ HCC). CK19 positivity was defined as membranous and/or cytoplasmic expression in $\geqslant$ 5% of tumor cells with moderate or strong intensity. Patients with other primary liver cancers, such as combined HCC and cholangiocarcinoma (cHCC-CC), intrahepatic cholangiocarcinoma (ICC) who received curative liver resection, were also included in the study. Clinical characteristics of $CK19^-$ HCC and $CK19^+$ HCC patients, including survival outcome after curative liver resection, were compared with that of cHCC-CC and ICC patients.

RESULTS: The overall survival (OS) rate of $CK19^-$ HCC (n = 49) after the curative surgical treatment was 90.7%, and 80.4% at 1 and 5 years after the resection. OS rate of $CK19^+$ HCC (n = 21) was 74.3%, 28.9% and OS rate of cHCC-CC (n = 22) was 66.7%, 32.2% at 1 and 5 years after the surgery. For ICC (n = 19), 1 and 5-year-OS rate was 50.2% and 14.3% after the curative resection. The OS rates of $CK19^+$ HCC and cHCC-CC were significantly lower than that of $CK19^-$ HCC, but higher than the OS rate of ICC (P = 0.000). There was no statistically significant difference in OS rate between $CK19^+$ HCC and cHCC-CC. The disease free survival (DFS) rate of $CK19^-$ HCC was 72.0% and 54.5% at 1 and 3 years after the surgical treatment. DFS rate of $CK19^+$ HCC was 53.3%, 34.3% and DFS rate of cHCC-CC was 51.5%, 39.2% at 1 and 3 years after the resection. For ICC, 1 and 3-year-DFS rate was 28.0% and 14.0% after the curative resection. DFS rate of $CK19^-$ HCC was significantly higher than that of ICC (P = 0.017), but marginally higher than DFS rate of either $CK19^+$ HCC or cHCC-CC (P = 0.097, P = 0.089, respectively). Predictors of outcome after the surgery of primary liver cancer were pathology of the resected mass, existence of microvascular invasion and accompanying satellite nodule.

CONCLUSION: Primary liver cancers with biliary components tended to show poorer surgical outcome. This suggested that immuno-phenotype of liver cancers was as important as their morphological classification.

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Key words: Cytokeratin 19; Hepatocellular carcinoma; Intrahepatic cholangiocarcinoma; Liver cancers; Hepatectomy

Peer reviewer: Min-Hsiung Pan, PhD, Professor, Department of Seafood Science, National Kaohsiung Marine University, No.142 Haijhuan Rd., Nanzih District, Kaohsiung 81143, Taiwan, China

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INTRODUCTION

Proposed treatment strategy for solid tumor is usually guided by tumor stage. In case of hepatocellular carcinoma (HCC), Barcelona Clinic Liver Cancer (BCLC) staging system, that has come to be widely accepted in clinical practice[1], carefully allocates HCC patients according to tumor stage and liver function as well as physical status. Despite the careful selection of candidates for each treatment option, HCC patients with similar BCLC grade that underwent the same mode of treatments may show very different outcome. It has been suggested that HCC heterogeneity in the molecular and morphological features may be related to the treatment outcome[2,3]. Recently, efforts have been made to classify HCC histopathologically, encompassing subpopulations of HCC according to their distinct molecular features[4].

While the World Health Organization (WHO) still classifies primary liver cancer into HCC, intrahepatic cholangiocarcinoma (ICC), and combined hepatocellular and cholangiocarcinoma (cHCC-CC)[5], there has been a report on HCCs whose cells of some proportion displayed cholangiolar differentiation and which showed highly aggressive behavior[6]. The authors claimed that this type of HCC with biliary differentiation must be different from cHCC-CC since it displayed typical HCC morphological features, and referred it as dual-phenotype HCC. On the other hands, according to the WHO classification, cHCC-CC is characterized by having unequivocal, intimately mixed elements of both HCC and ICC components within a single nodule[5], and it is also reported to show much worse prognosis than HCC[7-11].

Meanwhile, it has recently been proposed that HCC with biliary differentiation should be categorized as HCC with stem/progenitor cell immunophenotype whereas cHCC-CC should be included as mixed hepatobiliary carcinoma, classical type[4].

Since both HCC with biliary differentiation and cHCC-CC appeared to be the primary liver cancer displaying biliary phenotype, we hypothesized that the outcome of curative resection in primary liver cancer would be grave as the cancer expressed biliary phenotype rather than hepatic phenotype. In order to test this hypothesis, we compared the surgical outcome of classical HCC without biliary differentiation, with that of HCC with biliary differentiation, defined as having cytokeratin (CK) 19 positive cells, along with cHCC-CC and ICC.

MATERIALS AND METHODS

Case selection and histopathologic analysis

Liver specimens, surgically resected between January 2000 and August 2010 at Inha University Hospital (Incheon, South Korea) were reviewed and selected from pathologic files. Out of 204 patients who underwent liver resection due to HCC, 70 patients were assessed for CK19 positivity. These patients had well preserved liver function, evaluated by Child-Pugh scoring system. None of the selected cases experienced preoperative cancer treatment including transarterial chemoembolization (TACE). HCC was diagnosed under hematoxylin and eosin (HE) stain according to the morphologic criteria as defined by the WHO. Edmondson and Steiner Grading System (E-S, I-IV) was used to evaluate the degree of differentiation[5]. The primary antibody for CK19 (monoclonal, DAKO, Glostrup, Denmark, 1:50) was applied in order to detect CK19 positivity in HCC. CK19 positivity was defined as membranous and/or cytoplasmic expression in $\geqslant$ 5% of tumor cells with moderate or strong intensity.

Twenty-two cases of cHCC-CC were analyzed. Combined HCC-CC was diagnosed when there were evidences of both HCC and CC differentiation demonstrated within the same nodule[12]. Nineteen cases of ICC were included as the control.

Medical records of the selected patients were reviewed for etiology of underlying liver disease and laboratory data for liver function and tumor markers. The patients were regularly followed up. Liver function tests, acrylic fixed prosthesis measurements were done every 2-3 mo. Dynamic CT was regularly performed with the interval no longer than 6 mo. All patients were followed until the time of death or for at least 12 mo. Recurrence was defined as the appearance of a new lesion with the radiologic features of HCC. Early recurrence was defined as any recurrence within 12 mo after the initial hepatectomy.

Histopathologic analysis was also performed for tumor size, differentiation, presence of multiple tumors, microvascular and major vessel invasion, lymph node metastasis and existence of background liver cirrhosis.

Statistical analysis

Statistical analysis was performed using SPSS software version 12.0 (SPSS, Chicago, IL). Clinicopathologic characteristics among different liver cancers were compared using χ^2 test. Overall survival (OS) was calculated using Kaplan-Meier method, and differences in survival rate were compared using Log-rank test. Univariate analysis for factors affecting prognosis of primary liver cancer after curative resection was evaluated using Log-rank test. All variables that appeared to be associated with the prognosis were put under multivariate analysis using Cox proportional hazards model. Statistical significance and marginal significance were assumed when $P < 0.05$ and $P < 0.01$, respectively.

RESULTS

Pathological evaluation

Diagnosis of HCC was made under HE stain in all 70 cases. Forty-nine HCC patients had CK19 immunostain

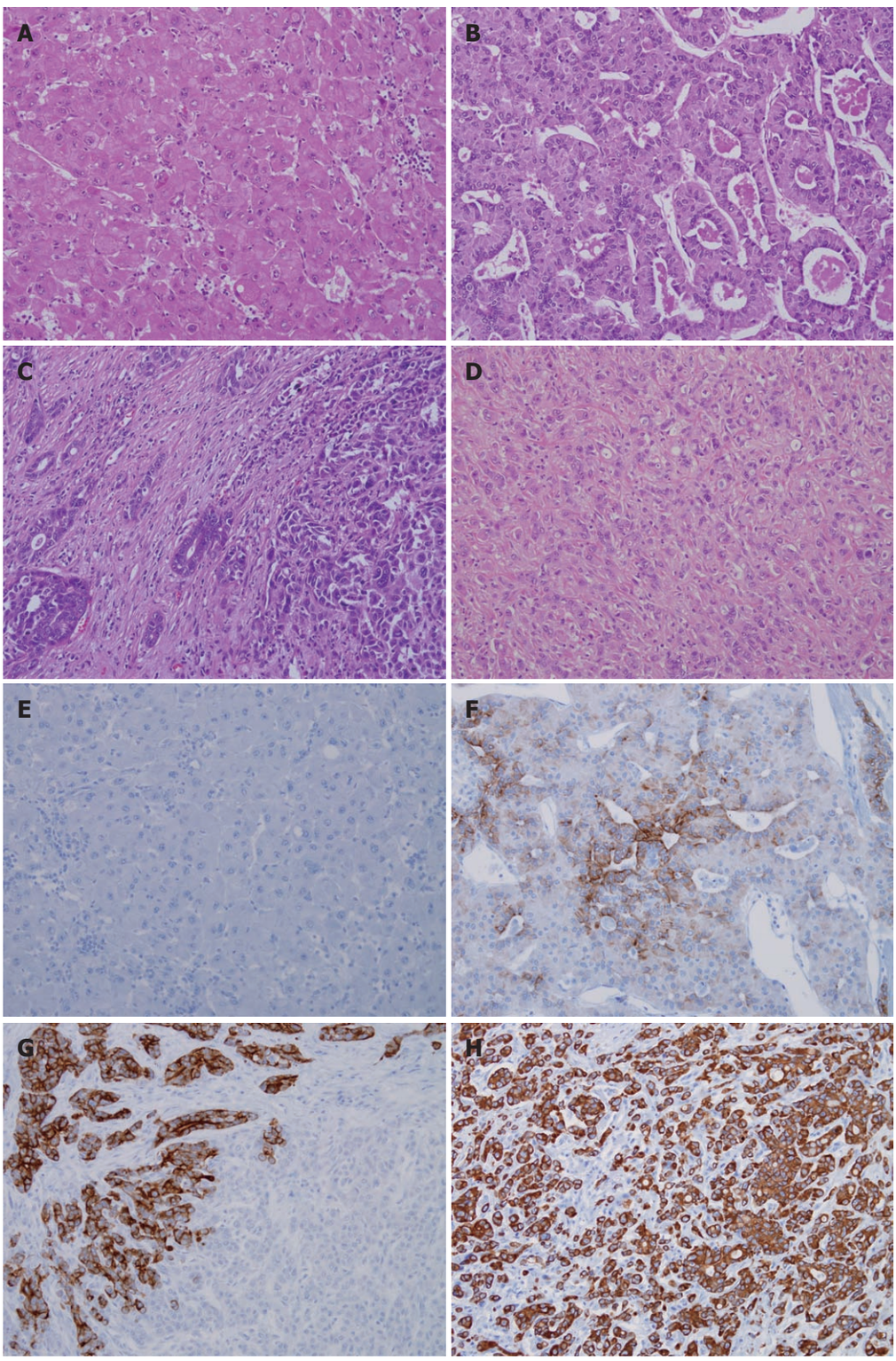


Figure 1 Pathological features of primary liver cancer. A-D: Hematoxylin and eosin (HE) stain for cytokeratin 19 negative hepatocellular carcinoma (CK19$^-$ HCC) (A), cytokeratin 19 positive hepatocellular carcinoma (CK19$^+$ HCC) (B), combined hepatocellular carcinoma and cholangiocarcinoma (cHCC-CC) and intrahepatic cholangiocarcinoma (ICC); E-H: Immunohistochemical stain results of CK19 stain for CK19$^-$ HCC (E), CK19$^+$ HCC (F), cHCC-CC (G) and ICC (H). All in original magnification × 200.

negative (CK19$^-$ HCC), and identified as HCC without biliary differentiation (Figure 1). Another 21 cases out of 70 HCC cases showed CK19 positivity (CK19$^+$ HCC) and classified as HCC with biliary differentiation (Figure 1).

Clinical features

All the patients underwent curative liver resection of primary liver cancer. There were no statistically significant differences in clinical findings of CK19$^-$ HCC (n = 49),

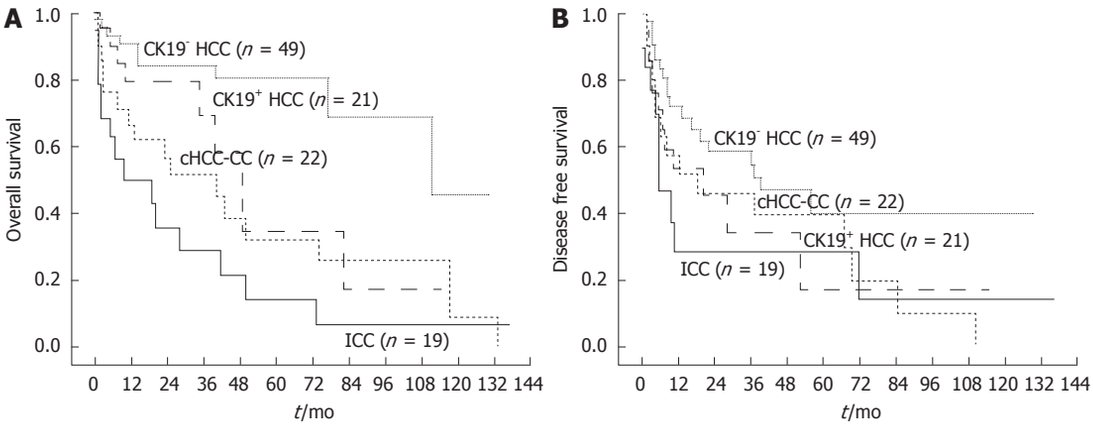


Figure 2 Clinical outcome after the curative liver resection of primary liver cancers. A: Overall survival (OS) rates of different groups of primary liver cancer after the curative resection. The OS rates of cytokeratin 19 positive hepatocellular carcinoma (CK19$^+$ HCC) and combined hepatocellular carcinoma and cholangiocarcinoma (cHCC-CC) were significantly lower than that of cytokeratin 19 negative hepatocellular carcinoma (CK19$^-$ HCC), but higher than the OS rate of intrahepatic cholangiocarcinoma (ICC) ($P < 0.05$). There was no statistically significant difference in OS rate between CK19$^+$ HCC and cHCC-CC ($P = 0.245$); B: Disease free survival (DFS) rate. The DFS rate of CK19$^-$ HCC was significantly higher than that of ICC ($P = 0.017$), but marginally higher than DFS rate of either CK19$^+$ HCC or cHCC-CC ($P = 0.097$, 0.089, respectively).

CK19$^+$ HCC (n = 21), cHCC-CC (n = 22) and ICC (n = 21) except in gender composition, etiologies of liver disease, and proportion of patients having liver cirrhosis (Table 1). In cases of CK19$^-$ and CK19$^+$ HCC, as well as cHCC-CC, male gender was the predominant population, whereas in ICC, there was no gender predisposition (Table 1). There was significantly lower number of patients with liver cirrhosis in the ICC group.

Comparison of clinical prognosis

The OS rate of CK19$^-$ HCC after the curative surgical treatment was 90.7% and 80.4% at 1 and 5 years after the resection, respectively. OS rate of CK19$^+$ HCC was 74.3%, 28.9%, and OS rate of cHCC-CC was 66.7%, 32.2% at 1 and 5 years after the surgery. In cases of ICC, 1 and 5-year-OS rate was 50.2% and 14.3% respectively after the curative resection. The OS rates of CK19$^+$ HCC and cHCC-CC were significantly lower than that of CK19$^-$ HCC, but higher than the OS rate of ICC (P = 0.000) (Figure 2A). There was no statistically significant difference in OS rate between CK19$^+$ HCC and cHCC-CC (Figure 2A).

The disease free survival (DFS) rate of CK19$^-$ HCC was 72.0% and 54.5% at 1 and 3 years after the surgical treatment. DFS rate of CK19$^+$ HCC was 53.3%, 34.3%, and DFS rate of cHCC-CC was 51.5%, 39.2% at 1 and 3 years after the resection. In cases of ICC, 1 and 3-year-DFS rate was 28.0% and 14.0% respectively after the curative resection. The DFS rate of CK19$^-$ HCC was significantly higher than that of ICC (P = 0.017), but marginally higher than DFS rate of either CK19$^+$ HCC or cHCC-CC (P = 0.097, P = 0.089, respectively) (Figure 2B). However there was no statistically significant difference in DFS rate among CK19$^+$ HCC, cHCC-CC and ICC (Figure 2B). The percentage of patients that received treatment for the recurred cancer was much smaller in cases of ICC whereas the majority of HCC regardless of CK19 expression, and cHCC-CC patients underwent TACE for the recurred mass (Table 1).

Prognostic factors for primary liver cancer after the curative resection

In cases of resectable primary liver cancer in functionally well preserved liver, surgical resection is usually recommended regardless of its pathologic diagnosis. Univariate analysis of prognostic factors after the curative resection in primary liver cancer was performed. The tested factors included pathologic diagnosis of the liver mass, age at the operation, gender, existence of liver cirrhosis, tumor size, tumor number, coexisting satellite nodule, existence of micro and macro vascular invasion, as well as lymph node and distant metastasis. Pathologic diagnosis of the resected mass, tumor size, accompanying satellite nodule, existence of microvascular, macrovascular and lymph node invasion were possible prognostic factors forecasting survival after the curative surgery (Table 2). On multivariate pathologic diagnosis of the resected mass, existence of microvascular invasion and accompanying satellite nodule affected the prognosis of primary liver cancer after the surgical resection (Table 2).

DISCUSSION

This study suggested that the outcome of the surgical treatment in the primary liver cancer would be worse as the resected tumor expressed more components with biliary phenotypes.

Although current WHO classification of primary liver cancer is still guided by the morphologic features of the tumor[13], new classification emphasizing morpho-phenotypic categories with patho-genetic implications is rapidly emerging[4]. Demand for the new type of primary liver cancer classification came from heterogeneous treatment outcome within the liver mass that had similar morphological features. Several studies supported that there would be subpopulation of HCCs, expressing bili-

Table 1 Clinical characteristics of patients and treatment modality of recurrent with primary liver cancer *n* (%)

	CK19⁻ HCC (*n* = 49)	CK19⁺ HCC (*n* = 21)	cHCC-CC (*n* = 22)	ICC (*n* = 19)	*P* value
Age (yr)					0.43
< 45	11 (22.4)	4 (19.0)	4 (18.2)	1 (5.3)	
⩾ 45	38 (77.6)	17 (81.0)	18 (81.8)	18 (94.7)	
Gender					0.014[a]
Male	41 (83.7)	14 (66.7)	18 (81.8)	9 (47.4)	
Female	8 (16.3)	7 (33.3)	4 (18.2)	10 (52.6)	
Underlying liver disease					0.004[a]
None	0 (0.0)	0 (0.0)	3 (13.6)	12 (63.2)	
HBV	38 (79.2)	18 (85.7)	17 (77.3)	2 (10.5)	
HCV	4 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	
Others	6 (12.5)	3 (14.3)	2 (9.1)	5 (26.3)	
Cirrhosis					0.024[a]
No	22 (44.9)	10 (47.6)	10 (45.5)	16 (84.2)	
Yes	27 (55.1)	11 (52.4)	12 (54.5)	3 (15.8)	
Tumor number					0.313
Single	41 (83.7)	17 (81.0)	16 (72.7)	18 (94.7)	
Multiple	8 (16.3)	4 (19.0)	6 (27.3)	1 (5.3)	
Satellite nodule					0.063
Absent	43 (87.8)	19 (90.5)	15 (68.2)	18 (94.7)	
Present	6 (12.2)	2 (9.5)	7 (31.8)	1 (5.3)	
Microvascular invasion					0.836
Absent	21 (42.9)	10 (47.6)	11 (55.0)	9 (47.4)	
Present	28 (57.1)	11 (52.4)	9 (45.0)	10 (52.6)	
Macrovascular invasion					0.119
Absent	45 (91.8)	17 (81.0)	19 (95.0)	14 (73.7)	
Present	4 (8.2)	4 (19.0)	1 (5.0)	5 (26.3)	
Lymph node metastasis					0.086
Absent	47 (95.9)	20 (95.5)	21 (95.5)	15 (78.9)	
Present	2 (4.1)	1 (4.8)	1 (4.5)	4 (21.1)	
Distant metastasis					0.521
Absent	46 (93.9)	21 (100)	21 (95.5)	17 (89.5)	
Present	3 (6.1)	0 (0.0)	1 (4.5)	2 (10.5)	
Differentiation					0.301
E1, E2 or well, moderate	15 (30.6)	7 (33.3)	9 (47.4)	9 (52.9)	
E3, E4 or poorly	34 (69.4)	14 (66.7)	10 (52.6)	8 (47.1)	
Treatment modality					
Number of patience with recurrence	19/49 (38.8)	12/21 (57.1)	15/22 (68.2)	11/19 (57.9)	
Conservative treatment	2/19 (10.5)	1/12 (8.3)	3/15 (20.0)	8/11 (72.7)	
Transarterial chemoembolization	15/19 (78.9)	11/12 (91.7)	12/15 (80.0)	0/11 (0.0)	
Systemic chemotherapy	0/19 (0.0)	0/12 (0.0)	0/15 (0.0)	2/11 (3.5)	
Others	2/19 (10.5)	0/12 (0.0)	0/15 (0.0)	3/11 (5.3)	

[a]$P < 0.05$ among 4 groups. CK19⁻ HCC: Cytokeratin 19 negative hepatocellular carcinoma; CK19⁺ HCC: Cytokeratin 19 positive hepatocellular carcinoma; cHCC-CC: Combined hepatocellular carcinoma and cholangiocarcinoma; ICC: Intrahepatic cholangiocarcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

ary phenotype as being CK19 positive, and that this subpopulation would demonstrate aggressive behavior[6,14-17]. Our study also showed that morphologically similar HCC might exhibit very different outcome after the curative treatment if their malignant cells contain components of biliary phenotype detected by CK19 immunochemical study.

Out of 70 patients who had CK19 immunochemical study, 21 patients had CK19 positive. These 70 patients underwent immunochemical study due to atypical pathologic features that made diagnosis ambiguous without immunochemical evaluation. Therefore, the proportion of CK19⁺ HCC was relatively higher than that of other studies[6,14]. Even though HCC patients in this study had resectable liver mass, they had higher Edmonson grade in more than 60% regardless of CK19 immunostain result. It is very likely that HCC with ambiguous morphology under HE stain might have poorer differentiation and thus might demonstrate less favorable surgical outcome. However, it appeared that CK19⁻ HCC had more favorable outcome compared with that of CK19⁺ HCC independent of Edmonson grade.

It is beyond the scope of this study addressing the origin of this biliary component in HCC. However, since CK19 is known as a stem cell marker, it can be contemplated that CK19 positive phenotype reflected an origin from a progenitor cell. Studies suggesting primary liver cancer of progenitor cell origin used CK19 as one of liver stem cell markers[18-22]. Moreover, one recent study reported that CK19 was well correlated with tumor aggressiveness, compared to other stemness-related markers[20,23]. In addition CK19 being a stem cell marker, a re-

Table 2 Univariate and multivariate analysis of predictors of survival

	Univariate analysis	Multivariate analysis		
Variable	*P* value	Hazard ratio	95% CI	*P* value
Pathologc diagnosis	0.000			0.000
CK19$^-$ HCC				
CK19$^+$ HCC		3.055	1.235-7.558	0.016
cHCC-CC		4.588	1.953-10.781	0.000
ICC		6.986	2.997-16.284	0.000
Age (yr)	0.672			
< 45 *vs* ≥ 45				
Gender	0.197			
Cirrhosis	0.542			
Tumor size (cm)	0.000			
< 5 *vs* ≥ 5				
Tumor number	0.179			
Single *vs* multiple				
Satellite nodule	0.001	1.997	1.010-3.949	0.047
Microvascular invasion	0.005	2.884	1.491-5.581	0.002
Macrovascular invasion	0.000			
Lymph node metastasis	0.002			
Distant metastasis	0.111			

CK19$^-$ HCC: Cytokeratin 19 negative hepatocellular carcinoma; CK19$^+$ HCC: Cytokeratin 19 positive hepatocellular carcinoma; cHCC-CC: Combined hepatocellular carcinoma and cholangiocarcinoma; ICC: Intrahepatic cholangiocarcinoma; 95% CI: 95% confidence interval.

port suggested that expression of CK19 was related with epidermal growth factor thereby increasing the growth abilities of HCC[24-26].

Most of the studies on CK19 in liver cancer performed immunohistochemical analysis in order to evaluate its expression[15-19]. Some studies that assessed *CK19* gene expression along with its protein expression demonstrated that protein expression well correlated gene expression[20,27].

Meanwhile, origin of cancer cells in cHCC-CC also needs to be verified. By definition, cHCC-CC has areas of typical HCC and areas of typical cholangiocarcinoma. It was reported that cholangiocarcinoma components in cHCC-CC were usually positive for CK19, and careful examination usually revealed intermediate morphology at the interface between HCC and cholangiocarcinoma areas[4].

Clinically, studies consistently reported that prognosis of cHCC-CC worse than that of HCC in general[7,8,21]. In this study, when CK19$^+$ HCC was compared with cHCC-CC, we found their clinical features somewhat similar, as both cell types demonstrated unfavorable prognosis than CK19$^-$ HCC. Regarding OS, both CK19$^+$ HCC and cHCC-CC showed lower OS rates than CK19$^-$ HCC, and higher rates than ICC. However, when we compared DFS rates, although CK19$^-$ HCC still showed higher DFS rate than any other types of primary liver cancer, there were no significant differences in DFS rates of CK19$^+$ HCC, cHCC-CC and ICC. This discrepancy in OS and DFS rates might be resulted from not having effective means of treating recurrent ICC when HCC and cHCC-CC could partly be controlled by the non-surgical method such as TACE.

Recently suggested histopathological classification of primary liver cancer put CK19$^+$ HCC as cancer of hepatocellular phenotype with stem/progenitor cell immunophenotype, whereas cHCC-CC as cancer of mixed hepatobiliary carcinoma, classical type. According to this proposed classification, these two subpopulations of liver cancer resided between the spectrum of HCC and ICC, where CK19$^+$ HCC closer to HCC and cHCC-CC being closer to cholangiocarcinoma[4]. In accordance with this classification, our study showed that clinical outcome of CK19$^+$ HCC and cHCC-CC after the curative liver resection was also placed between that of HCC and ICC where HCC side showing good prognosis and ICC side displaying poor prognosis. It seemed that primary liver cancer that possessed more components with biliary phenotype resulted in poor prognosis after the curative surgical treatment. However, since our study was limited by relatively small number of patients, especially in CK19$^+$ HCC and cHCC-CC group, another study over larger number of patients should be able to verify this suggestion.

COMMENTS

Background

Hepatocellular carcinoma (HCC) of similar clinical stage may produce heterogeneous treatment outcome, and immuno-phenotypical information of HCC might facilitate more appropriate stratification of the tumor.

Research frontiers

Efforts have been made to classify HCC histopathologically according to their distinct molecular features. Several studies suggested that there would be subpopulation of HCCs, expressing biliary phenotype as being cytokeratin (CK) 19 positive, and that this subpopulation would demonstrate aggressive behavior.

Innovations and breakthroughs

New classification emphasizing morpho-phenotypic categories with pathogenetic implications is rapidly emerging. This histopathological classification of primary liver cancer put cytokeratin 19 positive hepatocellular carcinoma (CK19$^+$ HCC) as cancer of hepatocellular phenotype with stem/progenitor cell immunophenotype, whereas combined hepatocellular and cholangiocarcinoma (cHCC-CC) as cancer of mixed hepatobiliary carcinoma, classical type. According to this proposed classification, these two subpopulations of liver cancer resided between the spectrum of HCC and intrahepatic cholangiocarcinoma, where CK19$^+$ HCC closer to HCC and cHCC-CC being closer to cholangiocarcinoma.

Applications

Primary liver cancer that possessed more components with biliary phenotype, detected by CK19 immunochemical study, might predict poor outcome after the curative surgical treatment. Therefore, alternative treatments or more vigorous adjuvant therapy after the surgical treatment could be recommended in such cases.

Terminology

Studies on primary liver cancer of progenitor cell origin used CK19 as one of liver stem cell markers along with other markers such as cluster of differentiation 133, c-kit and epithelial cell adhesion molecule. Among many stemness-related markers, a recent study reported that CK19 was well correlated with tumor aggressiveness.

Peer review

The authors examined three different types of primary liver cancers for its biliary phenotype and gave a vital conclusion that primary liver cancer that showed more of biliary components resulted in poor prognosis after the surgery. This work provides useful information on immuno-phenotype of primary liver cancers might be as important as their morphological classification with interesting results.

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BRIEF ARTICLE

Association of fucosyltransferase 2 gene variants with ulcerative colitis in Han and Uyghur patients in China

Ayinuer Aheman, He-Sheng Luo, Feng Gao

Ayinuer Aheman, He-Sheng Luo, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan University Medical School, Wuhan 430060, Hubei Province, China
Feng Gao, Department of Gastroenterology, People's Hospital of Xinjiang Uyghur Autonomous Region, Urumqi 830001, Xinjiang Uyghur Autonomous Region, China
Author contributions: Aheman A and Gao F contributed equally to this work, they designed the study, analyzed and interpreted the data, wrote and revised the manuscript; Luo HS acquired fund, conceived, designed and supervised the study, and revised the manuscript critically for important intellectual content; and all authors have read and approved the final version to be published.
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Correspondence to: He-Sheng Luo, Professor, Chief, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan University Medical School, Wuhan 430060, Hubei Province, China. xhnk@163.com
Telephone: +86-27-88041911 Fax: +86-27-88042292
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Abstract

AIM: To investigate the contribution of fucosyltransferase 2 (FUT2) variants to the genetic susceptibility and clinical heterogeneity of ulcerative colitis (UC) between Han and Uyghur patients in Xinjiang, China.

METHODS: A total of 102 UC patients (53 Han patients including 22 men and 31 women, and 49 Uyghur patients including 25 men and 24 women; aged 48 ± 16 years) and 310 age- and sex-matched healthy controls were enrolled from January 2010 to May 2011 in Xinjiang People's Hospital of China. UC was diagnosed based on the clinical, endoscopic and histological findings following Lennard-Jones criteria. Blood samples were collected and genomic DNA was extracted by the routine laboratory methods. Polymerase chain reaction-sequence-based typing method was used to identify FUT2 variants rs281377, rs1047781, rs601338 and rs602662. Genotypic and allelic frequencies were documented and compared between the UC patients and the healthy controls. Genotypic frequencies were also compared between Han and Uyghur patients. Potential association of genetic variation and UC between Han and Uyghur patients was examined.

RESULTS: rs281377 was found significantly associated with UC in the Han population as compared with the controls (P = 0.011) while rs281377 was not associated with UC in the Uyghur population (P = 0.06). TT homozygous rs281377 frequencies were higher in the UC groups than in the controls (88.7% *vs* 68.7% and 55.1% *vs* 50.3%). rs1047781 was specifically associated with UC in the Uyghur population (P = 0.001), but not associated with UC in the Han population (P = 0.13). TT homozygous rs1047781 frequencies were lower in the UC groups than in the controls (9.5% *vs* 11.8% and 4.0% *vs* 6.7%). rs601338 was statistically related to UC in both populations (Han, P = 0.025; Uyghur, P = 8.33 × 10^{-5}). AA homozygous rs601338 frequencies were lower in the UC groups than in the controls (0% *vs* 1.8% and 12.2% *vs* 13.4%). No association was found between rs602662 and UC in both Han and the Uyghur populations. Allelic analysis showed that rs281377 allele was significantly associated with UC in the Han population as compared with the controls [P = 0.001, odd ratio (OR) = 0.26], however, it was not associated with UC in the Uyghur population (P = 0.603, OR = 1.14), and rs1047781 allele was associated with UC in the Uyghur population (P = 0.001, OR = 0.029) while it was not associated with UC in the Han population (P = 0.074, OR = 0.62). Moreover, rs601338 was associated with UC in both Han (P = 0.005, OR = 0.1) and Uyghur populations (P = 0.002, OR = 0.43). Meta analysis showed that rs1047781 and rs601338 conferred risk of UC as compared with the controls [P = 0.005, OR = 0.47; P = 0.0003, OR = 0.35; 95% confidence interval (CI) = 0.31-0.72 and 0.21-0.58], but rs281377 and rs602662 showed no statistically significant differences between

patients with UC and controls (P = 0.10, OR = 0.71; P = 0.68, OR = 0.09; 95% CI = 0.47-1.07 and 0.56-1.47).

CONCLUSION: Functionally relevant *FUT2* gene variants are associated with UC, suggesting that they play a potential role in the pathogenesis of UC and may contribute to the clinical heterogeneity of UC between Han and Uyghur patients.

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Key words: Ulcerative colitis; Fucosyltransferase 2; Gene polymorphisms; Han; Uyghur

Peer reviewers: Sung-Gil Chi, Professor, School of Life Sciences and Biotechnology, Korea University, No. 301, Nok-Ji Building, Seoul 136-701, South Korea; Espen Melum, MD, Medical Department, Rikshospitalet University Hospital, Sognsvannsvn. 20, Oslo 0027, Norway

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease are often characterized as chronic inflammatory bowel diseases (IBD). Clinical features in both disorders include abdominal pain, diarrhea, weight loss and increased risk of developing colorectal cancer[1]. The etiology of UC is thought to be multifactorial, such as genetic, environmental, immune, and gut barrier factors[2]. Genetic factors involved in the regulation of the immune system are considered to play a significant role in the pathogenesis of IBD[3,4]. Much progress has been achieved in recent years regarding the genetic etiology of IBD. Studies have found that disease distribution and phenotypic appearance differ significantly between ethnic groups and even within populations. Apart from varying environmental factors that affect susceptible individuals, genetic heterogeneity between different populations itself plays an important role in IBD[5]. We previously compared the clinical characteristics of UC between the Han and Uyghur populations residing in the Xinjiang Uyghur Autonomous Region of China. We found differences between the Uyghur and Han populations living in the same region, where Uygur population had a higher prevalence of UC, a younger age of onset, an increased prevalence of the chronic persistence and acute outbreak type, more moderate and severe forms, a higher complication rate, and an increased frequency of positive anti-neutrophilic cytoplasmic antibodies[6,7]. However, the genetic heterogeneity related to UC between these two ethnic groups need to be investigated.

Secretor/non-secretor phenotypes [determined by the fucosyltransferase 2 (*FUT2*) gene] are associated with some metabolic and infectious diseases, and ABO glycosyltransferase activity has been shown to be involved in the pancreatic cancer risk[8]. Genetic variation in FUT2 has been implicated in susceptibility to *Helicobacter pylori*[9], Norovirus (Norwalk virus)[10,11], progression of human immunodeficiency virus[12], recurrent urinary tract infections[13], the development of vaginal candidiasis[14], gram-negative sepsis in infants[15] and cholera[16]. Carriers of non-secretor variants have higher plasma vitamin B12 levels than carriers of the secretor genotypes[17]. In addition to the genetic associations mentioned above, non-secretion of ABO blood group antigens into body fluids has been shown to be associated with rheumatic fever[18] and Crohn's disease, but not associated with UC[19].

Earlier studies have shown differences in the intestinal microbiota between the UC patients and healthy subjects[20] and alterations in the microbiota of IBD patients are related to changes in the FUT2 genotype[21]. The *FUT2* gene codes for an α (1,2)-fucosyltransferase and regulates the expression of ABH antigens in body secretions and the intestinal mucosa[22]. The *FUT2* gene is located on chromosome 19q13.3.4 (chromosome 19: 49, 199, 228-49, 209, 207) and consists of two exons. The cDNA is 3.1 kb long and encodes a polypeptide of 332 amino acid residues. The gene determines the secretion status of the ABO antigens with secretors having at least one functional FUT2 allele (Se), whereas non-secretors are homozygous for nonfunctional FUT2 allele[23]. rs601338 nonsense mutation (Trp143stop) in the *FUT2* gene of non-secretors has been reported in Caucasians (Europeans and Iranians) and Africans[24] and was found in approximately 1% of Chinese[25]. The frequency of non-secretors among east Asians is similar to Europeans and Africans, but east Asians are homozygous for a different weak-activity allele resulting from a rs1047781 missense mutation (Ile129phe)[26]. In Portuguese, two FUT2 polymorphisms, rs602662 and T839C, are associated with decreased or absent FUT2 enzyme activity[27]. rs281377 synonymous mutation (Asn-Asn) in the *FUT2* gene of secretors is found all over the world with a higher frequency than the wild type allele (Se)[28]. Four variants (rs281377, rs1047781, rs601338 and rs602662) in the *FUT2* genes have been identified in healthy Uyghur donors[29]. The rs1047781 was found in 54.7% of the Han populations with the most common alleles being rs281377, and rs281377 and rs1047781 were found in 94% of Chinese population[25]. rs281377 was reported in 71.2% and rs1047781 in 22.0% among the Uyghur populations[29]. In this study, we examined whether polymorphism of the *FUT2* gene differed between the Han and Uyghur patients with UC.

MATERIALS AND METHODS

Patients and controls

A total of 102 consecutive patients with UC (53 Han and 49 Uyghur, 47 men and 55 women, aged 18-78 years) and 310 age- and sex-matched healthy controls (161 Han and 149 Uyghur, 137 men and 173 women, aged 18-75 years) were enrolled from January 2010 to May 2011 in the De-

partment of Gastroenterology and the Physical Examination Center, Xinjiang People's Hospital of China. UC was diagnosed based on the clinical, endoscopic and histological findings following the Lennard-Jones criteria[30]. The extent of the disease was assessed by colonoscopy at the initial diagnosis and during follow-up. Extensive colitis was defined as lesions located beyond the splenic flexure. Distal colitis was defined as lesions limited to the region distal to the spleen flexure[31,32]. The study protocol was approved by the Ethics Committee of Xinjiang People's Hospital. Selected patients with UC and healthy controls are Chinese Han and Uyghur from Urumqi, Xinjiang, China. Informed consent was obtained from all the subjects.

Extraction of DNA

Genomic DNA was purified using a QIAamp DNA Blood Midikit (Qiagen, Germany). Purified genomic DNA from blood donors was stored frozen in micro-titre plates (10 ng/μL in TE buffer) until analyzed.

Polymerase chain reaction and sequencing primers

Primers for the polymerase chain reaction (PCR) amplification and sequencing were designed using conventional standard criteria. Primer specificity was checked with published nucleotide sequences using Basic Local Alignment Search Tool and analyzed for the risk of primer-dimer formation and secondary structures using Oligo 6.0 Software. The following pair of primers was used for the FUT2 (forward, 5'-AGCGCCCCGGGCCTCCATCTCC-3'; reverse, 5'-GGAACCATGTGTGCTTCTCATGCCCG-3'). The same forward and reverse primers were used for sequencing.

PCR and PCR product purification

Water of 17.3 μL, 0.5 μL 20 μmol/L forward primer, 0.5 μL 20 μmol/L reverse primer, 2.5 μL PCR buffer, 1 μL dNTPs, 2 μL $MgCl_2$ and 0.2 μL TaqGold (Applied Biosystems, Branchburg, New Jersey, United States) were added to 1 μL DNA (≥ 50 ng). Amplification conditions for the FUT2 PCR were: one cycle of 5 min at 95 ℃ followed by 10 cycles of 20 s at 95 ℃ and 40 s at 78 ℃ and 23 cycles of 10 s at 95 ℃, 10 s at 62 ℃ and 2 min at 72 ℃, and finally one cycle of 5 min at 72 ℃ and 4 ℃. The PCR was performed by a GeneAmp PCR system 9700 from the Applied Biosystems. PCR products were purified using the SAP-Exon method.

PCR product sequence

Twelve μL water, 1 μL 10 μmol/L forward primer, 2 μL BigDye sequencing buffer, 4 μL Bigdye (BigDye® Terminator v3.1Cycle Sequencing Kit) were added to 1 μL PCR product (≥ 50 ng). Cycle sequencing conditions for the FUT2 were: one cycle of 1 min at 96 ℃ followed by 25 cycles of 10 s at 96 ℃, 5 s at 50 ℃ and 4 min at 60 ℃ and 4 ℃. The PCR was performed by a GeneAmp PCR system 9700 from Applied Biosystems. DNA sequencing was performed using an automated sequencer (ABI 3730XL, Applied Biosystems). Chromas software version 2.0 was used to analyze the raw sequence data. The FUT2 genotyping method was established in accordance with the polymorphism (Figure 1). The frequencies of the genotypes were calculated by direct counting.

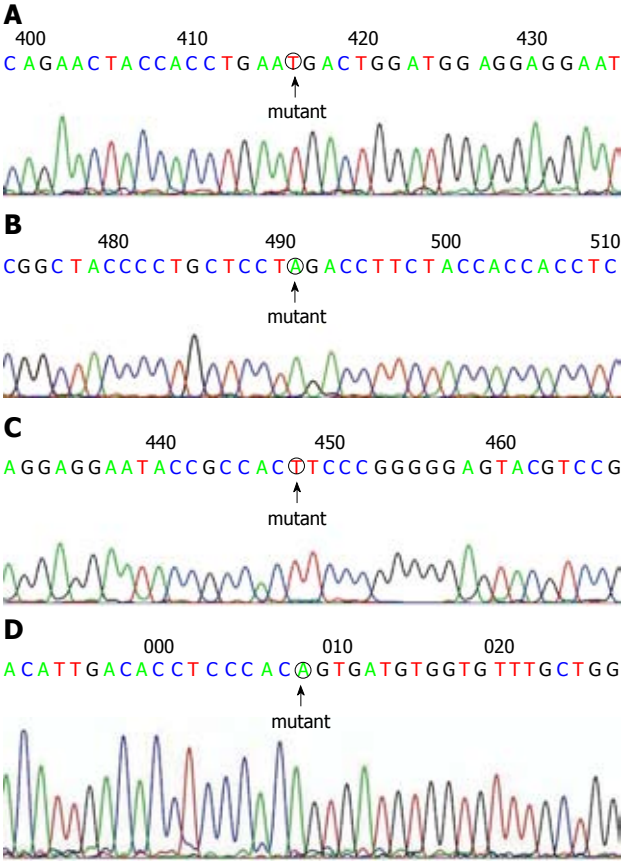


Figure 1 DNA sequence. A: Fucosyltransferase 2 (*FUT2*)-rs281377TT in a Han patient; B: FUT2- rs601338AA in a Uyghur patient; C: FUT2-rs1047781TT in a Han patient; D: FUT2-rs602662AA in a Uyghur patient.

Statistical analysis

Hardy-Weinberg disequilibrium was assessed with χ^2 test and clinical records including age were analyzed with *t* test. Allelic and genotypic association analyses were performed using the Pearson's 2 × 2 or 2 × 3 table χ^2 test. Odd ratio (OR) and 95% confidential interval (95% CI) were also calculated by Pearson's 2 × 2 χ^2 test. To adjust for the multiple tests performed and obtain an empirical null distribution of the association test *P* values (*P* empirical), we conducted 10 000 permutations in the case-control samples. Tests for the heterogeneity of effect size between Han and Uyghur populations were carried out using Cochrane's *Q* test, and $P < 0.05$ indicated that heterogeneity existed. When heterogeneity was found, random-effects model was applied using Cochran-Mantel-Hansel test to calculate the overall genetic effect of single nucleotide polymorphisms by combining the data from Han and Uyghur populations; otherwise, fixed-effects model was applied. The above analyses were performed using PLINK v.1.07 (http://pngu.mgh.harvard.edu/purcell/plink/).

Table 1 Clinical characteristics of the study subjects

Items	Han population	Uyghur population	Total
UC patients	53	49	102
Gender (male/female)	22/31	25/24	47/55
Age of onset (yr)	52 ± 15	46 ± 15	48 ± 16
Extensive colitis	20 (0.38)	30 (0.61)	50 (0.49)
Distal colitis	33 (0.62)	19 (0.39)	52 (0.51)
Mild and moderate	47 (0.89)	40 (0.82)	87 (0.85)
Severe	6 (0.11)	9 (0.18)	15 (0.15)
Healthy controls	161	149	310
Gender (male/female)	67/94	70/79	137/173
Age of onset (yr)	48 ± 15	49 ± 13	47 ± 14

Data are shown as mean ± SD or *n* (%). UC: Ulcerative colitis.

RESULTS

Clinical characteristics of UC patients

DNA was obtained from 102 consecutive UC patients and 310 healthy individuals who were well matched by sex and age. The main clinical characteristics of the UC patients are summarized in Table 1. UC patients included 47 women and 55 men with 53 Han and 49 Uyghur, and their average age at onset was 48 ± 16 years. Among the 310 healthy controls, there were 161 Han and 149 Uyghur including 137 men and 173 women, and their average age was 47 ± 14 years. There were no significant differences in sex ratio and the average age between the UC patients and healthy controls ($P = 0.74$ and $P = 0.801$).

Comparison of FUT2 genotype and allelic variants between UC patients and healthy controls

The genotype frequencies of the *FUT2* gene variants of the UC patients and the healthy controls are presented in Table 2. rs281377 was only associated with UC in Han population ($P = 0.011$), while rs1047781 was associated specifically with UC in Uyghur population ($P = 0.001$). rs601338 was statistically related to UC in both Han and Uyghur populations (Han, $P = 0.025$; Uyghur, $P = 8.33 \times 10^{-5}$). rs602662 was not associated with UC in both Han and Uyghur populations. TT homozygote of rs281377 was compared between the two ethnic UC groups and controls. Its frequencies were higher in the UC groups than in the controls (88.7% *vs* 68.7% and 55.1% *vs* 50.3%, respectively). TT homozygous rs1047781 frequencies (9.5% *vs* 11.8% and 4.0% *vs* 6.7%) and AA homozygous rs601338 frequencies (0% *vs* 1.8% and 12.2% *vs* 13.4%) were both lower than in the controls.

In allelic analysis as shown in Table 3 and Figure 2, rs281377 was found associated with UC only in the Han population ($P = 0.001$, OR = 0.26). rs1047781 was associated specifically with UC in Uyghur population ($P = 0.001$, OR = 0.29), whereas rs601338 was associated with UC in both Han and Uyghur populations (Han, $P = 0.005$, OR = 0.10; Uyghur, $P = 0.002$, OR = 0.43) and rs602662 was not associated with UC in both Han and Uyghur populations (Han, $P = 0.985$; Uyghur, $P = 0.652$, Figure 1).

The comparison between Han and Uyghur UC patients and controls revealed that genotype and allele rs281377 and rs601338 in the Han population were significantly associated with UC while in the Uyghur population, rs1047781 and rs601338 were significantly associated with UC.

Meta-analysis showed that rs1047781 and rs601338 conferred risk of UC as compared with the controls, ($P = 0.005$, OR = 0.47; $P = 0.0003$, OR = 0.35; 95% CI = 0.31-0.72 and 0.21-0.58) while rs281377 and rs602662 were not significantly different statistically between patients with UC and controls ($P = 0.100$, OR = 0.71; $P = 0.680$, OR = 0.90; 95% CI = 0.47-1.07 and 0.56-1.47).

DISCUSSION

UC is thought to fundamentally represent an altered interaction between the intestinal microbiota and the intestinal immune system[33]. The intestinal microbiota interacts with luminal enterocytes *via* host cell surface molecules, including oligosaccharides synthesized by glycosyltransferases[34]. Previous studies have shown that the allele polymorphism with a synonymous mutation rs281377, including the nonsense mutation rs601338, and missense mutation rs1047781 and rs602662, is almost inactive and is responsible for some instances of non-secretor statu[27]. Non-secretor phenotypes in the population occurring as the absence of particular carbohydrate molecules in the mucosa may have conferred protection against some pathogens. It is demonstrated that Fut2-null mice do not express fucosyl glycolipid FGA1 in the colon, whereas normal mice do[35]. Commensal bacteria and probiotics may exert their protective effects *via* preventing adherence, or even displacing pathogenic bacteria[36], which is consistent with the notion that FUT2 and non-secretor may affect the status of the gastrointestinal microbiota. Furthermore, changes in the microflora of IBD patients have been documented.

Our study indicated that the genetic polymorphisms of the *FUT2* gene were correlated with UC, and the associations differed between Han and Uyghur people in China. We analyzed four variants of *FUT2* gene, the frequency of three variants of the *FUT2* genotype (*rs281377*, *rs1047781* and *rs601338*) showed significant associations with UC ($P < 0.05$), however, the associations differed between the two ethnic groups. In the Han population, genotype and allele of rs281377 and rs601338 were significantly associated with UC whereas in the Uyghur population, genotype rs1047781 and rs601338 were associated with UC.

We also compared the TT homozygote of rs281377 between the two ethnic UC groups and controls. Its frequencies were higher in the UC groups than in the controls (88.7% *vs* 68.7 and 55.1% *vs* 50.3%, respectively). TT homozygous rs1047781 (9.5% *vs* 11.8 and 4.0% *vs* 6.7%) and AA homozygous rs601338 frequencies (0% *vs* 1.8% and 12.2% *vs* 13.4%) were both lower than in the controls.

A limitation of the present study was the relatively

Table 2 Genotypic association of four single nucleotide polymorphisms with ulcerative colitis in Han and Uyghur populations *n* (%)

SNPs	Allele	Groups	UC cases	Controls	χ^2	*P*
rs281377	C/T	Han	0 (0)/6 (11.3)/47 (88.7)	9 (5.6)/42 (26.1)/110 (68.4)	9.091	0.011
	C/T	Uyghur	10 (20.4)/12 (24.5)/27 (55.1)	15 (10.1)/59 (39.6)/75 (50.3)	5.633	0.060
rs1047781	T/A	Han	5 (9.5)/13 (24.5)/35 (66.0)	19 (11.8)/61 (37.9)/81 (50.3)	4.077	0.130
	T/A	Uyghur	2 (4.0)/4 (8.2)/43 (87.8)	10 (6.7)/50 (33.6)/89 (59.7)	13.480	0.001
rs601338	A/G	Han	0 (0)/1 (1.9)/52 (98.1)	3 (1.8)/23 (14.3)/135 (83.9)	7.382	0.025
	A/G	Uyghur	6 (12.2)/9 (18.4)/34 (69.4)	20 (13.4)/76 (51.0)/53 (35.6)	18.790	8.33×10^{-5}
rs602662	A/G	Han	0 (0)/3 (5.7)/50 (94.3)	0 (0)/9 (5.6)/152 (94.4)	NA	NA
	A/G	Uyghur	5 (10.2)/15 (30.6)/29 (59.2)	11 (7.4)/61 (40.9)/77 (51.7)	1.776	0.412

UC: Ulcerative colitis; SNPs: Single nucleotide polymorphisms; NA: Not available.

Table 3 Allelic association of four single nucleotide polymorphisms with ulcerative colitis in Han, Uyghur and combined populations

SNPs	Groups	Risk allele	Frequency (case/control)	χ^2	Pobs	Pemp	OR (95% CI)	*P* (*Q*)	Poverall	OR (95% CI)
rs281377	Han	C	0.057/0.186	10.290	0.001	0.002	0.26 (0.11-0.63)	0.003	0.100	0.71 (0.47-1.07)
	Uyghur	C	0.327/0.299	0.270	0.603	0.636	1.14 (0.70-1.86)			
rs1047781	Han	T	0.217/0.308	3.203	0.074	0.106	0.62 (0.37-1.05)	0.100	0.005	0.47 (0.31-0.72)
	Uyghur	T	0.082/0.235	10.950	0.001	0.002	0.29 (0.13-0.63)			
rs601338	Han	A	0.009/0.090	7.954	0.005	0.102	0.10 (0.01-0.72)	0.140	0.0003	0.35 (0.21-0.58)
	Uyghur	A	0.214/0.389	9.979	0.002	0.002	0.43 (0.25-0.73)			
rs602662	Han	A	0.028/0.028	0.000	0.985	1.000	1.01 (0.27-3.81)	0.860	0.680	0.90 (0.56-1.47)
	Uyghur	A	0.255/0.279	0.204	0.652	0.699	0.89 (0.53-1.49)			

SNPs: Single nucleotide polymorphisms; Pobs: Observed *P* value, was calculated by $2 \times 2\ \chi^2$ test; Pemp: Pempirical, empirical null distribution of the association test *P* values; *P*(*Q*): Heterogeneity test, was calculated by Cochrane's *Q* test; Poverall: Meta-analysis *P* value, was calculated by Mantel–Haenszel test under fixed-effects or random-effects model; OR: Odd ratio; 95% CI: 95% confidential interval.

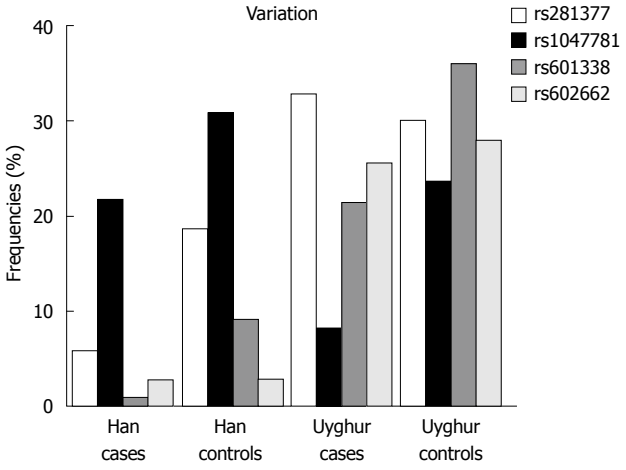


Figure 2 Allelic analysis of fucosyltransferase 2 variants and ulcerative colitis in Han and Uyghur populations.

small number of patients included. In the subgroup analysis, population stratification may lower the sufficient number of correlation analysis and weaken the significance of our results. In order to overcome these inadequacies and confirm our results, further research on a larger number of participants will be needed in the future.

In conclusion, our study found that the functional FUT2 variants might not only play a role in the pathogenesis of UC, but also contribute to the different clinical manifestations of UC between Han and Uyghur patients in north-west China. The effects of the different genotypes on the intestinal microbiota of normal controls and patients with UC are still unknown and likely represent a potentially productive area for future research in the quest to better understand the pathogenesis and treatment of UC.

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COMMENTS

Background

Earlier studies have shown differences in the intestinal microbiota between the ulcerative colitis (UC) patients and healthy subjects, and alterations in the microbiota of inflammatory bowel diseases (IBD) patients are related to changes in the fucosyltransferase 2 (*FUT2*) genotype. In this study, the authors examined whether polymorphism of the *FUT2* gene differed between the Han and Uyghur patients with UC.

Research frontiers

FUT2 and non-secretor may affect the status of the gastrointestinal microbiota. Studies have found that disease distribution and phenotypic appearance differ significantly between ethnic groups and even within populations. The associations of the genetic heterogeneity of UC between two ethnic groups, Chinese Han and Uyghur, were investigated.

Innovations and breakthroughs

This study found that the functional FUT2 variants might not only play a role in the pathogenesis of UC, but also contribute to the different clinical manifestations of UC between Han and Uyghur patients in North-West China.

Applications

The effects of the different genotypes on the intestinal microbiota of normal controls and patients with UC are still unknown and likely represent a potentially productive area for future research in the quest to better understand the pathogenesis and treatment of UC.

Terminology

UC is a form of IBD. It is a refractory, chronic, and nonspecific disease which usually occurs in the rectum and the entire colon; FUT2 encodes the α (1,2) fucosyltransferase that determines blood group secretor status, and synthesizes the H-type 1 antigen in saliva and mucosa.

Peer review

The author investigated the contribution of FUT2 variants to the genetic susceptibility and clinical heterogeneity of UC between Han and Uyghur patients. This manuscript contains potentially interesting findings.

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BRIEF ARTICLE

Emergency transcatheter arterial embolization for patients with acute massive duodenal ulcer hemorrhage

Yong-Li Wang, Ying-Sheng Cheng, Li-Zhen Liu, Zhong-Hui He, Kun-Hong Ding

Yong-Li Wang, Li-Zhen Liu, Zhong-Hui He, Kun-Hong Ding, Department of Interventional Radiology, Fengxian Branch Hospital of Affiliated 6th People's Hospital of Shanghai Jiaotong University, Shanghai 201400, China
Ying-Sheng Cheng, Department of Diagnostic and Interventional Radiology, Shanghai Jiaotong University Affiliated 6th People's Hospital, Shanghai 200233, China
Author contributions: Wang YL performed all interventional radiology procedures in all patients with acute massive duodenal ulcer hemorrhage, and he also designed and wrote the manuscript; Cheng YS gave some good suggestions about how to design, write and polish the manuscript; Liu LZ and He ZH assisted Wang YL to carry out all patients' management plans, and undertook collection of all patients' material; and Ding KH was a nurse working in digital subtraction radiography, who participated in all patients' management procedures and collected patient information.
Correspondence to: Dr. Ying-Sheng Cheng, MD, PhD, Assistant Professor, Department of Diagnostic and Interventional Radiology, Shanghai Jiaotong University Affiliated 6th People's Hospital, Shanghai 200233, China. cjr.chengysh@vip.163.com
Telephone: +86-21-57426335 Fax: +86-21-57416150
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Abstract

AIM: To evaluate the efficacy and safety of emergency transcatheter arterial embolization (ETAE) for patients with acute massive duodenal ulcer hemorrhage.

METHODS: Twenty-nine consecutive patients with acute massive bleeding of duodenal ulcer were admitted to our hospital from 2006 to 2011. Superselective angiography of the celiac and gastroduodenal arteries was performed to find out the bleeding sites before ETAE, then, embolotherapy was done with gelatin sponge particles or microstrips *via* a 5 French angiographic catheter or 3 French microcatheter. After ETAE, further superior mesenteric arteriography was undertaken in case collateral circulation supplied areas of the duodenal ulcer. Technical and clinical success rates were analyzed. Changes in the mucous membrane were observed using endoscopy following ETAE.

RESULTS: Angiography showed active bleeding with extravasation of contrast medium in seven cases with a 24% positive rate of celiac artery bleeding, and in 19 cases with a 65.5% rate of gastroduodenal artery bleeding. There were no angiographic signs of bleeding in three patients who underwent endoscopy prior to ETAE. Twenty-six patients achieved immediate hemostasis and technical success rate reached 90%. No hemostasis was observed in 27 patients within 30 d after ETAE and clinical success rate was 93%. Recurrent hemorrhage occurred in two patients who drank a lot of wine who were treated by a second embolotherapy in the same way. Five patients underwent transient ischem with light abdominal pain under xiphoid, spontaneous restoration without special treatment. No mucous necrosis happened to 29 cases for ischem of gastroduodenal arteries embolized.

CONCLUSION: ETAE is an effective and safe measure to control acute massive bleeding of duodenal ulcer.

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Key words: Transcatheter embolization; Massive bleeding; Duodenal ulcer; Angiography

Peer reviewer: Mototsugu Kato, MD, Department of Endoscopy, Hokkaido University Hospital, Nishi-5, Kita-14, Kita-ku, Sapporo 060-8648, Japan

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INTRODUCTION

Acute massive hemorrhage of duodenal ulcer is a common emergency. Emergency endoscopic hemostasis for duodenal ulcer is regarded as the first-choice therapy and considered to be the gold standard treatment[1-3]. However, endoscopic therapy does not successfully control bleeding or rebleeding in 8%-25% of patients[4-6]. Surgical treatment is a second choice after failure of endoscopic hemostatic therapy, and improves efficacy in these patients, but there is no decrease in mortality rate[7]. Most patients, especially, treated by endoscopy but rebleeding occurred, are physically weak, the indications of life are not stable, thus it is difficult to receive a second endoscopic or surgical treatment. Transcatheter arterial embolization has been performed for at least two to three decades and has been shown to be effective at controlling hemorrhage and decreasing mortality[7,8-12]. With the development of interventional radiology, emergency transcatheter arterial embolization (ETAE) has also been used for treatment of massive bleeding of duodenal ulcer because it is minimally invasive, repeatable, effective and safe for patients with advanced age, weakness and unstable blood pressure[3,13,14].

MATERIALS AND METHODS

Materials

We collected and retrospectively analyzed data from 29 consecutive patients with acute massive bleeding of duodenal ulcer, and a transfusion requirement of at $\geqslant$ 4 units of packed red blood cells per 24 h[3,15-17], who were admitted to our hospital from January 2006 to August 2011. There were 18 male and 11 female patients, aged 16-67 years, with an average age of 36 years. All cases were diagnosed by endoscopy, and 10 patients received endoscopy, before receiving angiography and artery embolization, which clearly indicated the existence of duodenal ulcer. Electrocoagulation was carried for treatment, including three patients who suffered from rebleeding after surgery, and then ETAE was conducted. Nineteen patients presented with massive bleeding or hemodynamic instability (hypotension with systolic pressure < 100 mmHg or clinical shock secondary to blood loss) that did not respond to conservative treatment combined with volume replacement and proton pump inhibitors. They were sent directly from the emergency room for interventional radiological angiographic diagnosis and ETAE. Three to five days after treatment, endoscopic diagnosis clearly indicated the presence of duodenal ulcer once again. Six to eleven units of blood were transfused before treatment and 2-4 units were transfused after ETAE, which aimed to correct severe anemia, instead of correcting blood volume and restoring blood pressure.

Operating methods and procedures of ETAE

Under digital subtraction angiography, we used the improved Seldinger technique to puncture the right or left femoral artery, inserted a 6 F arterial catheter sheath (Japan Terumo), for elderly patients with significant tortuosity of the femoral artery, and used a 6 F anti-bending catheter sheath to open the tortuous blood vessels, to facilitate the superselective intubation of the catheter. We used a 5 F Rosch or Yasiro radiography catheter to conduct celiac artery angiography, and a 0.035-inch ultrasmooth black guide wire, to superselect the radiography catheter for gastroduodenal artery angiography. For patients with significant tortuous vessels, w used a coaxial catheter technique to superselect the 3F SP microcatheter (Japan Terumo), under the support of the radiography catheter, for angiography of the gastroduodenal artery or its branches. For branch arteries with clear bleeding or for the bleeding branch arteries that the microcatheter could reach, we used the microcatheter technique. Microcatheters were used for embolization of 17 patients, 14 of whom underwent embolization of bleeding the branch arteries, and three of whom underwent embolization of duodenal arteries; 5 F catheters were used for 12 patients. After the bleeding sites were identified, the catheter or microcatheter was placed in the proximal end of the bleeding artery, and then gelatin sponge particles or sponge strips were injected *via* the catheter or microcatheter. The specifications of the sponge particles were: 1 mm × 1 mm × 1 mm; the specification of the sponge strips were 5 mm × 1 mm × 1 mm. Under continuous fluoroscopy, we used contrast agent to inject the embolic agent, stop injecting the sponge particles or the sponge strips once the contrast agent in corresponding bleeding artery or bleeding gastroduodenal artery slow flow or the contrast agent is held up. Superior mesenteric artery angiography should be carried out after gastroduodenal artery embolization, to prevent the establishment of collateral arterial blood supply of the branches from the superior mesenteric artery: the pancreaticoduodenal anterior inferior arcuate artery and pancreaticoduodenal posterior inferior arcuate artery. If collateral blood supply is found by radiography, there is a possibility of rebleeding, and arterial embolization must be carried out using a microcatheter. Conservative treatment is recommended after embolization: to give drugs for inhibition of gastric acid secretion and gastric mucosal protective agents and hemostasis, and inject fluid to correct hypovolemia. For patients who did not have preoperative endoscopy, 3-5 d after bleeding stopped, when they had stable blood pressure and partial recovery from weakness, we carried out endoscopy and *Helicobacter pylori* (*H. pylori*)-related examination. Patients who were *H. pylori*-positive were treated with oral antibacterial medication. Before 2008, follow-up was by outpatient visit and letter, and after that time, follow-up was by outpatient visit and telephone interview for 3-6 mo.

Evaluation of efficacy and safety

The bleeding artery after ETAE is occluded by angiography, or contrast agent extravasation disappears, and the blood pressure is stable; no further bleeding occurs within 30 d after ETAE, and the occult blood test of fecal within the 3-6 mo follow-up period is negative. The

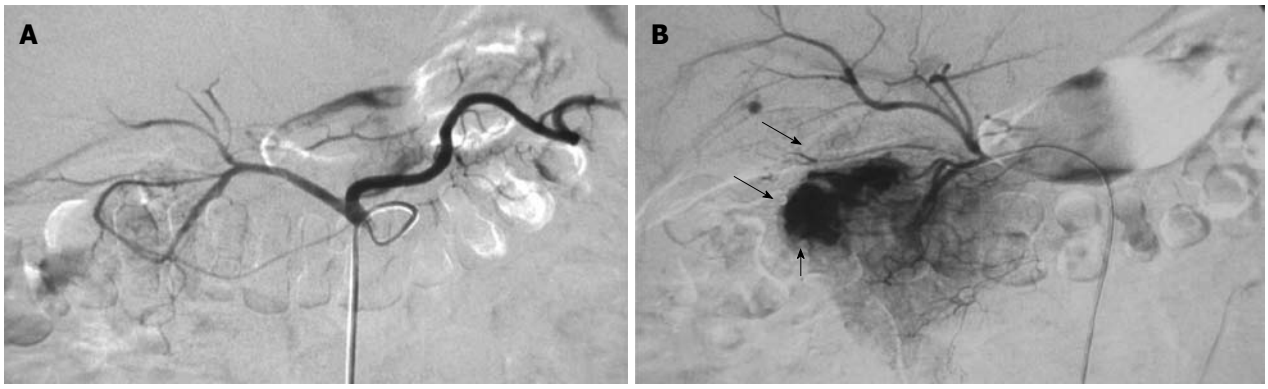


Figure 1 Impact of catheter position on the signs of bleeding. A: When the catheter tip was in the celiac artery, radiography failed to show signs of bleeding in the gastroduodenal artery; B: When the catheter super-select to the beginning site of the gastroduodenal artery for angiography, and the exo-contrast agent and smearing of the duodenal mucosa (arrows) are shown.

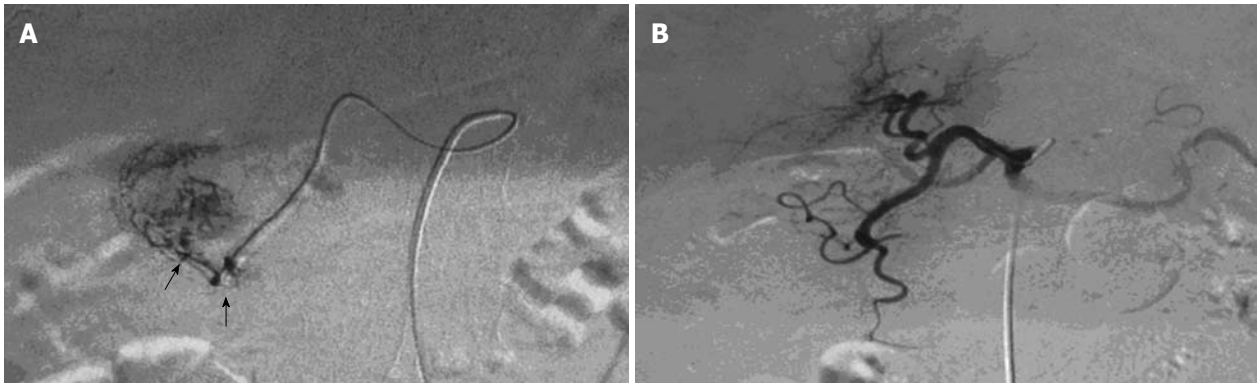


Figure 2 Angiographic changes before and after embolization of the gastroduodenal branch by microcatheter. A: Micro-catheter super-select to the gastroduodenal artery branch for angiography, and the exo-contrast agent is shown (indicated by the arrows); B: After embolization by micro-catheter, angiography showed that the bleeding had stopped; the distal end of the bleeding artery was kept open, to avoid widespread ischemia of normal tissue.

serious complications of ETAE are duodenal infarction or ischemic necrosis perforation, which are indicated by persistent upper abdominal (xiphoid) pain, peritoneal irritation, or muscle guarding sign.

Note for vascular grading: the 1st-grade-artery is abdominal aortic artery, the 2nd-grade-artery is celiac artery, the 3rd-grade-artery is gastroduodenal artery, and the 4th-grade-artery is branch arteries of gastroduodenal artery (the grading of blood vessels is defined by the authors, pending further discussion).

RESULTS

Among the 29 patients, there were three cases with multiple organ failure and seven elderly patients. Angiography with a celiac artery catheter (opening of the 2nd grade vessel) found bleeding in seven cases, with a positive rate of 24%; gastroduodenal arteriography (opening of the 3rd grade vessel) found signs of bleeding in 19 patients, with a positive rate of 66%; and there were three cases with no significant bleeding, with a negative proportion of 10%. Manifestations of hemorrhage were as follows: angiography of most celiac aorta hemorrhage was shown as the contrast agent gathering in small areas or with no imaging; angiography of gastroduodenal artery bleeding was shown as the contrast agent gathering into a lake-like area, with a large amount smearing the duodenal wall (Figures 1 and 2). There were 26 cases of immediate hemostasis after ETAE, with a technical success rate of 90%. The three cases that showed no significant escape of contrast agent on angiography were not included in the calculation of the technical success rate. Twenty-nine patients had stable blood pressure after ETAE. Four patients had a hemoglobin concentration of 4.5-5.0 g/L due to a large amount of bleeding before ETAE, and to correct anemia, 2-4 units of red blood cell suspension were infused. The amount of red blood cell suspension infused was significantly lower compared to 4-11 units before ETAE. The clinical success rate was 93% (27/29), and there were only two cases of rebleeding due to ulcers caused by heavy drinking. Angiography was carried out again and treated with the same procedure for embolization. After 3-6 mo follow-up, there was only one case of recurrence, and ETAE treatment was conducted once again. The first angiography of all the 3 cases of rebleeding showed positive bleeding.

Five patients had showed transient upper abdominal pain due to acute ischemia after ETAE, and the symptoms relieved spontaneously without treatment. There was no occurrence of persistent upper abdominal pain,

muscle guarding and other peritoneal irritation caused by severe gastroduodenal artery infarction, ischemia, or perforation.

Nineteen patients received endoscopy after ETAE. Seven of the 10 patients who had received endoscopy before ETAE received endoscopy again after treatment. Endoscopy showed that the normal duodenal mucosa in the embolization area showed pale change, and there was no case of necrosis. We distinguished complicated mucosal defects from previous duodenal ulcer by histological representations under endoscopic examination, which showed that complicated mucosal defects were not only around the areas near the duodenal ulcer but also far removed from them, distributed along the embolized artery.

Twenty-eight cases of duodenal ulcer were certainly related to *H. pylori* infection, and only in one case could the cause of duodenal ulcer not be identified. All patients who were *H. pylori*-positive took oral antibacterial medication during follow-up.

DISCUSSION

Efficacy of medical conservative therapy for upper gastrointestinal hemorrhage caused by duodenal ulcer is poor. Endoscopy and endoscopic hemostasis is the recognized method for first-line treatment[1-3,18,19]. The incidence of rebleeding after initial endoscopic treatment is about 14.1%, and in 15%-25% of cases, the bleeding cannot be controlled endoscopically or rebleeding requires alternative treatment[20,21], and surgery has become such an alternative[22,23]. However, for elderly patients with other associated diseases[24], and patients after surgery, rebleeding is a poor prognostic factor for massive hemorrhage of duodenal ulcer, with mortality of 44%-60%[3]. In recent years, interventional radiology has played an increasingly important role in the emergency treatment of the disease. Angiography cannot only quickly and clearly identify the bleeding site, but ETAE has become an effective alternative treatment for patients with recurrence after endoscopic and surgical treatment[12,25]. It has even been reported that ETAE can be the preferred treatment for emergency gastrointestinal hemorrhage[7,26,27].

The efficacy of interventional treatment is largely influenced by the treatment devices and methods. From the mid-1980s to mid-1990s, the diameter of the catheter used ranged between 5 and 6 F, and the texture of the catheter was coarse. Therefore, it was difficult to carry out super-selective catheterization, which meant that most patients with gastroduodenal bleeding due to arterial rupture used transcatheter perfusion of vasopressin to shrink arterial lesions, to stop the bleeding. However, local perfusion of drugs still achieved a good technical success rate. Miller *et al*[28] have reported that there were 267 patients who received perfusion therapy between 1979 and 1984; the success rate was 70%-80%, but the rebleeding rate was still nearly 20%, and for perfusion patients with difficulty in hemostasis, the rebleeding rate was up to 40%. The reason was that some elderly patients had hypertension, coronary atherosclerosis, or myocardial infarction and other underlying diseases, even partial use of vasopressin, its usage amount and continuous drug use time were still limited. In addition, the rich collateral circulation still provide much more blood supply for the bleeding duodenal intestinal. In recent years, with improvements of the technical skills of catheter, guide wire and other devices and the extensive use of coax system of micro-catheter (2.4-2.6 French size)[29], and arterial intubation by super-selection to the 3rd-grade or more above and the embolization have been skillfully used in most units, which has ensured the accuracy and effectiveness of ETAE, and has ensured blood supply of normal tissues. From our ETAE results of 29 patients, the success rate and clinical success rate were 90% and 93%, respectively. The results basically agreed with the technical success rate of 95% and clinical success rate of 52%-95% reported previously for ETAE treatment of upper gastrointestinal hemorrhage[29,30]. Earlier reports have shown that only two of seven cases of embolization had rebleeding, and four of six patients with conservative treatment had rebleeding. Toyoda *et al*[31] have reported control of bleeding after endoscopic treatment of 11 patients with duodenal ulcer. The bleeding was stopped in 10 patients treated with ETAE, with an efficacy of 91%. The remaining patient had atherosclerosis and embolization could only be applied to the proximal gastroduodenal artery, and as the embolization of the distal artery has established collateral circulation which led to rebleeding.

The patients with hemorrhage of gastroduodenal ulcer who had been clearly diagnosed by endoscopy and treated showed intermittent bleeding, while angiography could not clearly indicate the signs of bleeding, and there were three such patients in this group. Are such patients treated with ETAE? Although they were not included in calculating the technical success rate of ETAE, we believe that stopping bleeding or negative performance in angiography is only an illusion, which is related to low blood volume, vasoconstrictor drugs, or short-term thrombosis caused by hemostatic agents. If not handled, once the blood pressure is corrected, the contracted blood vessel expands again, or thrombolysis occurs, and rebleeding occurs. Under such circumstances, ETAE should still be applied[13], and the three patients treated by ETAE achieved clinical success.

For patients with multiple organ failure, although there were only two cases in this group of patients, randomized controlled studies could not be carried out. ETAE treatment effectively controlled massive bleeding caused by arterial rupture, and both of the two cases achieved technical and clinical success. There were another seven elderly patients, and only one had rebleeding after ETAE treatment, due to arteriosclerosis and tortuous vessels. Only the proximal gastroduodenal artery was embolized, and the branch of the superior mesenteric artery - pancreatic collateral arteries was open. Hemostasis was achieved after super-selective embolization of the collateral branch of the superior mesenteric artery. At the same time, the patient's situation reminded us that angiography

of the superior mesenteric artery can be used as a routine procedure before ETAE, by which rebleeding caused by collateral circulation of the duodenum can be avoided. In general, second endoscopic treatment, subsequent reoperation and unfavorable prognosis do not adversely affect the effective implementation of ETAE; on the contrary, ETAE can be repeatedly used for partial treatment of rebleeding due to its minimally invasive nature. It can be rapidly implemented because there is no need for preoperative preparation, and it can effectively control bleeding because it is a real-procedure and it accurately shows the bleeding site.

From the results of this group of patients, we can conclude that the treatment of duodenal massive bleeding using ETAE is safe, without severe ischemic complications such as duodenal infarction[32,33]. After ETAE treatment, some patients showed short-term duodenal ischemic symptoms, xiphoid pain or secret anguish, but these were relieved spontaneously without treatment. We initially selected, without experience, small particles as embolic agents, with a diameter of 300-500 μm. This resulted in mucosal defects far removed from the immediate area of the duodenal ulcer, which were distributed extensively along the embolized artery. For a few patients, endoscopy after ETAE showed that there were mild ischemic changes in the duodenal mucosa, manifested as the normal mucosa surrounding the ulcers was pale, but there were no ulceration and necrosis of normal mucosa after arterial embolization. Literature reported 28 patients with gastroduodenal distal artery embolized, and 7 cases had duodenal stenosis caused by ischemia, and for the 29 cases with arterial proximal embolized, only two cases showed the aforementioned pathological changes. The reason is related to mixed use of various embolic agents, including liquid embolic agents, at the same as ETAE treatment. There were 32 cases reported in another group, ETAE caused two cases of duodenal necrosis and one of new ulcer formation[34]. This was related to the use of gelatin sponge powder. Both liquid embolic agents and gelatin sponge powder can cause damage to the submucosal microvascular network, which can lead to loss of the compensatory blood supply of the mucosa.

We used large gelatin particles or strips of gelatin sponge for embolization of the proximal gastroduodenal artery. even the embolization of small bleeding artery is also under the premise that the bleeding targeting is a very clear, to implement the micro-catheter superselective ETAE, that is on the two levels of the diameter of embolic agent and embolization range, to ensure the submucosal microvascular network is not damaged, thus the complications reported in the literature did not occur. In terms of safety, we do not agree with the approach proposed by Lee *et al*[35] in choosing the type of embolic agents. They have used the liquid embolic agent N-butyl-2-cyanoacrylate to embolize 16 patients, and two patients showed a large area of thrombosis and multiple gastric ulcers. Therefore, choosing embolic agents of the right size and superselective catheterization and controlling the embolization range can guarantee ETAE safety.

In conclusion, ETAE is an effective, safe and fast emergency procedure for the treatment of massive bleeding of duodenal ulcer, which is especially suitable for frail elderly patients or those with multiorgan dysfunction with bleeding duodenal ulcer.

COMMENTS

Background

Acute massive hemorrhage of duodenal ulcer is a life-threatening condition. Emergency endoscopic hemostasis for duodenal ulcer is regarded as first-line therapy and considered to be the gold standard treatment. Surgery is usually considered for secondary intervention after failure of endoscopic hemostasis, or rebleeding. Transcatheter endovascular embolization is an alternative method of surgical treatment for stopping bleeding.

Research frontiers

Emergency transcatheter arterial embolization (ETAE) is an alternative to hemostasis after failure of endoscopic treatment. In the area of hemostasis for massive bleeding of duodenal ulcer, the research hotspot is to evaluate the efficacy and success rate of ETAE, and whether it becomes a first-line therapy.

Innovations and breakthroughs

Transcatheter arterial embolization has become a mature and effective method of hemostasis for arterial bleeding, but it is still controversial whether it can become the first choice for massive gastrointestinal hemorrhage. It has been successful in treating peptic ulcer bleeding. The innovations of this study were that the authors adopted the supra-selected arterial angiography to increase the positive rate of diagnosis and identify sites of ulcer bleeding. They also used it as the first-line treatment for some particular patients, such as older patients, the extra-weakness and the unstable-pressure with acute massive ulcer hemorrhage.

Applications

The results suggest that ETAE *via* supraselection angiography is an effective and safe therapy and could be an alternative, even first-choice therapy, for endoscopic hemostasis in some patients with acute massive hemorrhage of duodenal ulcer.

Terminology

Transcatheter arterial embolization: In peptic ulcer, trauma and other injury to the arterial system results in arterial rupture and massive hemorrhage, a catheter is inserted into the target artery, and contrast agent is injected to locate the ruptured vessel. Other agents can then be injected *via* the catheter to block the artery.

Peer review

This was a case series of 29 patients who received ETAE for acute massive hemorrhage of duodenal ulcer. The success rate of hemostasis was 90%. Recurrent hemorrhage occurred in two patients. Severe complications were not found. In conclusion, transcatheter arterial embolization is an effective and safe procedure to control acute massive bleeding of duodenal ulcer. It has some minor problems.

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BRIEF ARTICLE

Diagnosis of gastric intraepithelial neoplasia by narrow-band imaging and confocal laser endomicroscopy

Shu-Fang Wang, Yun-Sheng Yang, Li-Xin Wei, Zhong-Sheng Lu, Ming-Zhou Guo, Jin Huang, Li-Hua Peng, Gang Sun, En-Qiang Ling-Hu, Jiang-Yun Meng

Shu-Fang Wang, Yun-Sheng Yang, Zhong-Sheng Lu, Ming-Zhou Guo, Jin Huang, Li-Hua Peng, Gang Sun, En-Qiang Ling-Hu, Jiang-Yun Meng, Departments of Gastroenterology and Hepatology, Chinese PLA General Hospital, Beijing 100853, China

Li-Xin Wei, Departments of Pathology, Chinese PLA General Hospital, Beijing 100853, China

Author contributions: Wang SF designed the study and wrote the manuscript; Wang SF, Yang YS, Lu ZS, Guo MZ, Sun G, Huang J, Peng LH, Ling-Hu EQ and Meng JY performed the majority of the experiments and collected all the necessary human material; Wei LX performed all the pathological diagnoses; and Yang YS provided financial support for this work and was also involved in the editing of the manuscript.

Correspondence to: Yun-Sheng Yang, Professor, Departments of Gastroenterology and Hepatology, Chinese PLA General Hospital, No. 28 Fuxing Road, Beijing 100853, China. sunny301ddc@126.com
Telephone: +86-10-55499307 Fax: +86-10-68154653

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Abstract

AIM: To evaluate the diagnosis of different differentiated gastric intraepithelial neoplasia (IN) by magnification endoscopy combined with narrow-band imaging (ME-NBI) and confocal laser endomicroscopy (CLE).

METHODS: Eligible patients with suspected gastric IN lesions previously diagnosed by endoscopy in secondary hospitals and scheduled for further diagnosis and treatment were recruited for this study. Excluded from the study were patients who had liver cirrhosis, impaired renal function, acute gastrointestinal (GI) bleeding, coagulopathy, esophageal varices, jaundice, and GI post-surgery. Also excluded were those who were pregnant, breastfeeding, were younger than 18 years old, or were unable to provide informed consent. All patients had all mucus and bile cleared from their stomachs. They then received upper GI endoscopy. When a mucosal lesion is found during observation with white-light imaging, the lesion is visualized using maximal magnification, employing gradual movement of the tip of the endoscope to bring the image into focus. Saved images are analyzed. Confocal images were evaluated by two endoscopists (Huang J and Li MY), who were familiar with CLE, blinded to the related information about the lesions, and asked to classify each lesion as either a low grade dysplasia (LGD) or high grade dysplasia (HGD) according to given criteria. The results were compared with the final histopathologic diagnosis. ME-NBI images were evaluated by two endoscopists (Lu ZS and Ling-Hu EQ) who were familiar with NBI, blinded to the related information about the lesions and CLE images, and were asked to classify each lesion as a LGD or HGD according to the "microvascular pattern and surface pattern" classification system. The results were compared with the final histopathologic diagnosis.

RESULTS: The study included 32 pathology-proven low grade gastric IN and 26 pathology-proven high grade gastric IN that were detected with any of the modalities. CLE and ME-NBI enabled clear visualization of the vascular microsurface patterns and microvascular structures of the gastric mucosa. The accuracy of the CLE and the ME-NBI diagnosis was 88% (95% CI: 78%-98%) and 81% (95% CI: 69%-93%), respectively. The kappa coefficient of agreement between the histopathology and the *in vivo* CLE imaging was 0.755; between the histopathology and the *in vivo* CLE imaging was 0.615. McNemar's test (binomial distribution used) indicated that the agreement was significant ($P < 0.05$). When patients were diagnosed by ME-NBI with CLE, the overall accuracy of the diagnosis was 86.21% (95% CI: 73%-96%), and the kappa coefficient of agreement was 0.713, according to McNemar's test ($P < 0.05$).

CONCLUSION: Higher diagnostic accuracy, sensitivity

and specificity of CLE over ME-NBI indicate the feasibility of these two techniques for the efficacious diagnostic classification of gastric IN.

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Key words: Gastric intraepithelial neoplasia; Histological diagnosis; Confocal laser endomicroscopy; Magnification endoscopy; Narrow-band imaging; Gastric intraepithelial neoplasia lesion

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Wang SF, Yang YS, Wei LX, Lu ZS, Guo MZ, Huang J, Peng LH, Sun G, Ling-Hu EQ, Meng JY. Diagnosis of gastric intraepithelial neoplasia by narrow-band imaging and confocal laser endomicroscopy. *World J Gastroenterol* 2012; 18(34): 4771-4780 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i34/4771.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i34.4771

INTRODUCTION

Gastric cancer is one of the most common malignant diseases of the gastrointestinal (GI) tract. Substantial interest has arisen in recent years in the diagnosis and management of early gastric cancer because of the high cure rate achieved with treatment of these lesions. Diagnosis and localization of early gastric cancer is problematic because of the lack of any endoscopic characteristics, even with multiple random biopsies.

In recent years, endoscopic image quality has improved as the devices have advanced technologically, including chromoendoscopy, high resolution and magnification endoscopy[1]. Additionally, magnification endoscopy combined with narrow-band imaging (ME-NBI) has been used to achieve endoscopic pathology in gastric disease.

Although these techniques improve the visualization and detection of mucosal lesions, a biopsy of the targeted lesion must still be performed for a formal histological diagnosis of cellular and architectural atypia. However, there are several disadvantages that may be associated with biopsies or endoscopic resection, including bleeding and perforation. Non-representative biopsies may miss relevant portions of tissue, leading to an underestimation of the diagnosis. Random biopsy sampling or endoscopic resection for non-neoplastic lesions can also be time-consuming. It would be ideal if a definite diagnosis could be made during endoscopy without the need for a biopsy.

Recent technological advances in miniaturization have allowed for a confocal scanning microscope to be integrated into a conventional flexible endoscope, or into trans-endoscopic probes, a technique now known as confocal endomicroscopy (CEM) or confocal laser endomicroscopy (CLE)[2-4]. This newly-developed technology has enabled endoscopists to collect real-time *in vivo* histological images, or "virtual biopsies", of the GI mucosa during endoscopy, and has stimulated significant interest in the application of this technique in clinical gastroenterology.

MATERIALS AND METHODS

Patients

Patients with suspected gastric intraepithelial neoplasia (IN) lesions previously diagnosed by endoscopy in secondary hospitals and scheduled for further diagnosis and treatment were recruited for this study from September 2008 to July 2009 at the General Hospital of the People's Liberation Army, Beijing, China.

Excluded from the study were patients who had liver cirrhosis, impaired renal function, acute GI bleeding, coagulopathy, esophageal varices, jaundice, and GI post-surgery. Also excluded were those who were pregnant, breastfeeding, were younger than 18 years old, were unable to provide informed consent, or had a known allergy to fluorescein sodium. The General Hospital of the People's Liberation Army's clinical research ethics committee approved the protocol, and written informed consent was obtained from all participants.

NBI and magnified endoscopy system

The magnifying video endoscope used in this study was a GIF-H260Z (Olympus Medical Systems Co, Tokyo, Japan).

The videoendoscope is mounted on the charge coupled device (CCD) positioned on the tip of the endoscope as the imaging sensing device, with a xenon lamp used as the light source. The system is based on a black and white CCD, in which color separation is achieved through the use of a red-green-blue (RGB) rotary filter wheel equipped within the light source unit. The RGB filter consists of three broadband optical filters and covers all wavelengths of the visible spectrum, ranging from approximately 400 to 800 nm.

The NBI system uses two narrow band illuminations of 415 nm and 540 nm by NBI filter. Under NBI observation, the broadband white light derived from the xenon lamp splits into two bands (wavelength of 415 nm and 540 nm) and illuminates the surface of the mucosa. In the RGB sequential system, the NBI filter is placed on the light path in front of the RGB rotary filters. When observing with the NBI filter, the light illumination passes only B and G filters to obtain 415 nm and 540 nm images. Then, 415 nm is allocated to B and G channels so that blood vessels on the mucosal surface are reproduced in a brownish color, and 540 nm is allocated to the R channel so that the vessels in the deeper layer can be indicated in a greenish-blue color.

CLE system

All procedures were performed using a confocal laser endomicroscope (EC-3870K; OptiScan, Notting Hill, Australia and Pentax Corp., Tokyo, Japan)[5].

The confocal microscope integrated into the conventional endoscope collects images at a scan rate of 1.6 frames per second (1024 × 512 pixels) or 0.8 frames per second (1024 × 1024 pixels) with an adjustable scanning depth ranging from 0 to 250 μm, a field of view of 475 μm × 475 μm, a lateral resolution of 0.7 μm, and an axial resolution of 7 μm.

NBI and magnified endoscopic procedure

It is important to clear all mucus and bile from the stomach before upper GI endoscopy. Patients were given the following mixture 30 min before the endoscopic procedure: 100 mL of water with 10 000 units of pronase (Kaken Pharmaceutical, Tokyo, Japan), 1 g of sodium bicarbonate, and 10 mL of dimethylpolysiloxane (20 mg/mL, Horii Pharmaceutical Ind., Osaka, Japan).

A soft black hood (MB-162 for GIF-Q240Z, or MB-46 for GIFH260Z and GIF-Q160Z; Olympus, Tokyo) is essential for optimal magnification endoscopy. Prior to the NBI examination, the hood is mounted on the tip of the endoscope to enable the endoscopist to consistently fix the mucosa at a distance of approximately 2 mm so that maximal magnification of the endoscopic image can be obtained.

Before detailed inspection, adherent mucus is removed by water flushing. After the endoscope was inserted, the entire stomach was initially observed with conventional white light to exclude obvious lesions. At least 35 photographs were taken from the entire stomach, even if there were no obvious lesions.

Once a mucosal lesion is found during observation with white-light imaging, it is then visualized using maximal magnification, employing gradual movement of the tip of the endoscope to bring the image into focus, with the distally attached soft black hood being used to stabilize the tip of the endoscope onto the tissue. Instead of pressing the tip of the hood, gentle suction should be applied, without causing mucosal injury, and the lesion is then observed by magnification endoscopy with NBI. The images were saved to a specific folder for later analysis.

CLE procedure

After observation *via* ME-NBI, fluorescent contrasts should be used for CEM[6] (such as intravenously delivered fluorescein or topically administered acriflavine) and deployed through a spraying catheter.

Intravenously delivered fluorescein distributes throughout the extracellular matrix of the surface epithelium and lamina propria, but does not stain cell nuclei. Topically administered acriflavine stains cell nuclei of the surface epithelium, but does not penetrate to deeper layers of the GI mucosa. Acriflavine is a mutagenic dye and a potential human carcinogen, which limits its clinical utility.

Fluorescein is usually administered within 5 min of imaging; however, the dose and timing of contrast administration have not been standardized. After the contrast administration, the tip of the confocal endomicroscope or miniprobe is positioned in gentle contact with the area of interest to obtain high-resolution confocal images. Accumulated images can be saved for post-procedural analysis.

Histologic assessment

The resected specimens were fixed in 4% methanal, embedded in paraffin, sectioned into 4 mm thick samples, and stained with hematoxylin-eosin for routine histopathologic examination. Slides were coded and examined blindly by two pathologists (Wang YL and Wei LX), who were blinded to the endomicroscopic result, with diagnoses being confirmed by both individuals. The histological diagnostic criteria were based on the WHO classification. Early gastric cancer is defined as a malignant tumor limited to the mucosa or submucosa irrespective of the presence of lymph node metastases[7,8]. This includes the two types of low-grade neoplasia/dysplasia (LGD) and high-grade neoplasia/dysplasia (HGD), which are categorized according to the modified Vienna classification[9]. Early gastric cancer is further classified into two major groups: differentiated early gastric cancer which has tubular or papillary structures resembling the metaplastic intestinal epithelium; and undifferentiated early gastric cancer, which includes poorly differentiated carcinoma, and is characterized by discretely proliferating tumor cells with sparse or no tubular formation[10]. In the images of magnification endoscopy with NBI, an irregular microvascular pattern (MVP) and/or an irregular microsurface pattern (MSP), together with a clear demarcation line, are the hallmarks of early gastric cancer.

Evaluation of confocal images

All the confocal laser endoscopy images were stored in a specific folder, with the quality of each image being scored as good (no moving artifacts, gastric pit structure and vessel can be identified clearly), average (artifacts present but gastric pit and vascular architecture can be recognized), and poor (artifacts do not allow judgment of the image). The images were evaluated by two endoscopists (Huang J and Li MY) who were familiar with CLE, blinded to the related information about the lesions, and asked to classify each lesion as either a LGD or HGD according to the aforementioned criteria. The results were compared with the final histopathologic diagnosis.

Evaluation of ME-NBI images

All the NBI magnified images were stored in a specific folder. The images were evaluated by two endoscopists (Lu ZS and Ling-Hu EQ) who were familiar with NBI, blinded to the related information about the lesions and CLE images, and asked to classify each lesion as either a LGD or HGD according to the "microvascular pattern (V) and surface pattern (S)" classification system[11]. The results were compared with the final histopathologic diagnosis.

Statistical analysis

The statistical analysis was performed using the statistical software package SPSS 13.0 (SPSS, Chicago, IL, United States). The accuracy, sensitivity and specificity of the

CLE and ME-NBI diagnosis were respectively calculated in comparison with the histopathologic diagnosis.

RESULTS

Study population

A total of 59 patients participated in this study. The mean (± SD) age of the participants was 63.1 years, and the study population consisted of 44 male and 15 female patients.

In CLE, the 36 patients with low grade gastric intraepithelial neoplasia (LIN) included 9 women (25%) and 27 men (85%), with an average age of 60 years (range: 35-85 years). The 23 patients with high grade gastric intraepithelial neoplasia (HIN) showed significant male predominance as well, with 17 men (74%) and 6 women (23%). The average age was 68 years (range: 45-84 years).

A total of 1912 CLE images obtained from 62 lesions were used for analysis. The mean duration of the CLE procedure for each patient was 19 min (range: 11-30 min). After CLE, all patients developed a slight yellow coloration of the skin and urine, which disappeared in all cases within 24 h. No severe side-effects were noted.

In ME-NBI, the 45 patients with LIN included 12 women (27%) and 33 men (73%), with an average age of 63 years (range: 40-84 years). The 14 patients with HIN showed significant male predominance as well, with 11 men (79%) and 3 women (21%). The average age was 65 years (range: 36-85 years). The endoscopic and pathologic features of all the lesions are summarized below.

A total of 688 ME-NBI images obtained from 62 lesions were used for analysis. The mean duration of the ME-NBI procedure for each patient was 17 min (range: 10-25 min).

CLE characteristics

Gastric fundus: Superficial lamella gastric pits are round. Few pits compose one unit. The edge crack is the line type. Net-like subepithelial capillary network patterns surround the gastric pits (Figure 1A).

Gastric body: The gastric pits are round. Honeycomb-like subepithelial capillary network patterns surround the gastric pits (Figure 1B).

Gastric antrum: The gastric pits are line type. Coil-shaped subepithelial capillary network patterns surround the gastric pits (Figure 1C).

Gastric IN

LIN showed that, for the different sizes of gastric pits, all capillary networks are thickening and circuitous (Figure 2A); HIN showed an abnormal arrangement of gastric pits. The thickening capillary network and the increasing branch present a mass shape (Figure 2B).

ME-NBI characteristics

Gastric fundus: S: The individual morphology of the crypt epithelium shows a uniformly round/oval structure. Few structures are composed of one unit separated from the others. The width and length of the crypt epithelium are constant. The arrangement and distribution are regular and symmetrical; V: The mucosal capillaries have a uniform shape that can be closed-loop (polygonal). They have a consistent size and their arrangement and distribution are regular and symmetrical. Superficial lamella gastric pits are round (Figure 3A).

Gastric body: S: The individual morphology of the crypt epithelium shows a uniformly round structure. The width and length of the crypt epithelium are constant. The arrangement and distribution are regular and symmetrical; V: The mucosal capillaries have a uniform shape that can be closed-loop (polygonal). They have a consistent size and their arrangement and distribution are regular and symmetrical (Figure 3B).

Gastric antrum: S: The individual morphology of the crypt epithelium shows a uniformly round/linear structure. The width and length of the crypt epithelium are constant. The arrangement and distribution are regular and symmetrical; V: The mucosal capillaries have a uniform shape that can be open-loop. They have a consistent size and their arrangement and distribution are regular and symmetrical (Figure 3C).

LIN presented irregular surface and MVPs with a clear demarcation line (Figure 4A). HIN presented irregular surface and MVPs with a demarcation line. Few areas showed as flat (mean absent MSP) with an obscured demarcation line (Figure 4B).

Irregular surface pattern: The individual morphology of the crypt epithelium shows an irregular tubular/linear/curved/papillary/villous structure. The width and length of the crypt epithelium varies. Arrangement and distribution are irregular and asymmetric.

Irregular MVP: The vessels differ in shape, being closed-loop (polygonal), open-loop, tortuous, branched, bizarrely-shaped, and with or without a network. The size of the vessels also varies and their arrangement and distribution are irregular and asymmetrical.

Comparative data in CLE imaging and histopathology

Among 58 patients with gastric IN, 22 lesions with HIN and 29 lesions with LIN were diagnosed by CLE. The results are summarized in Table 1. The sensitivity and specificity of CLE for predicting low grade gastric IN were 90.63% and 84.62%, respectively. The overall accuracy of the CLE diagnosis was 88% (95% CI: 78%-98%), and the kappa coefficient of agreement between the histopathology and the *in vivo* CLE imaging was 0.755. McNemar's test (binomial distribution used) indicated good agreement between the final histopathology and the CLE imaging ($P < 0.05$).

Comparative data in ME-NBI imaging and histopathology

Among 58 patients of gastric IN, 20 lesions with HIN and 27 lesions with LIN were diagnosed by ME-NBI.

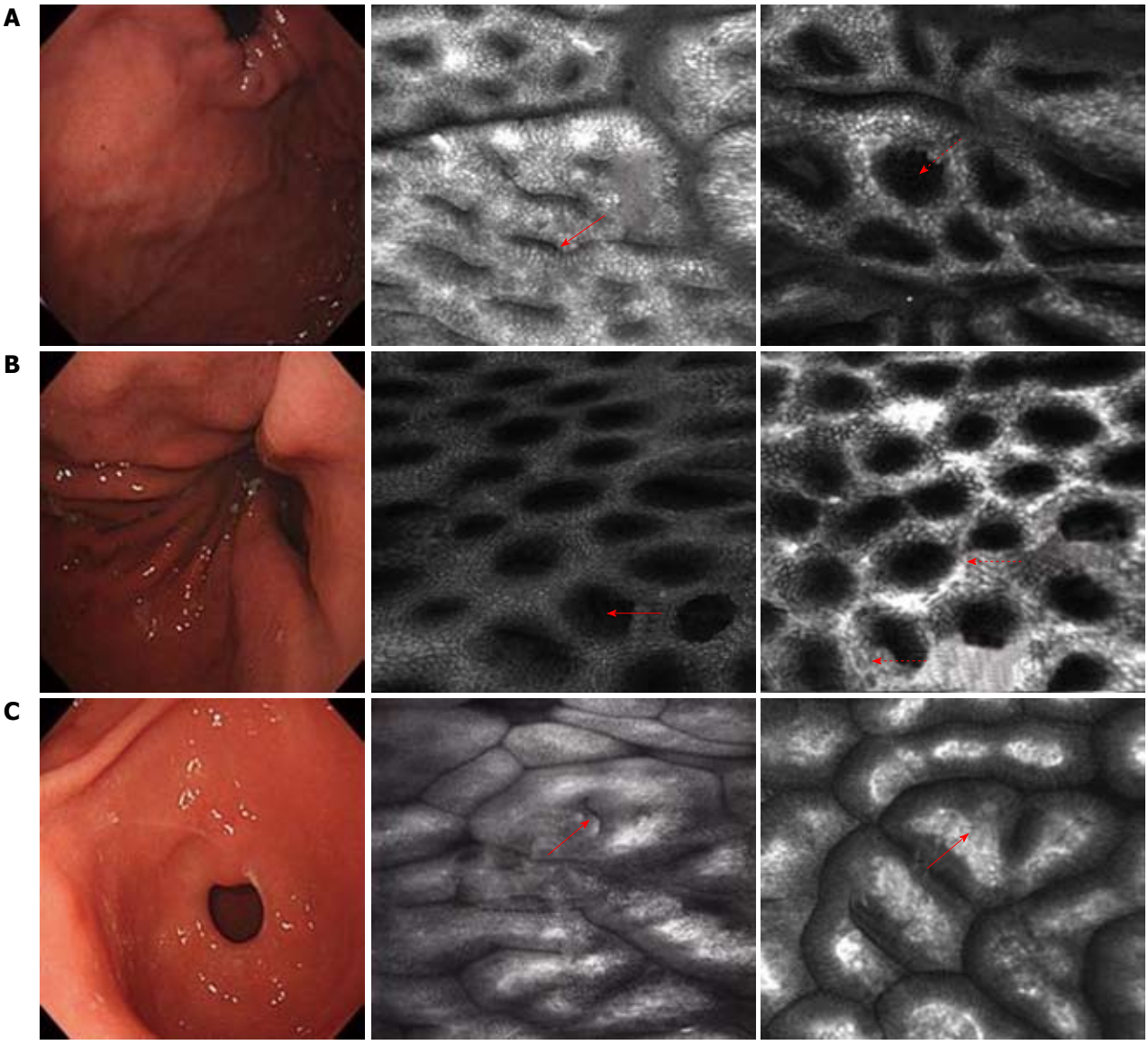


Figure 1 Routine endoscopy and confocal laser endomicroscopy images. A: Gastric fundus: Superficial lamella gastric pits are round (solid arrow). Few pits compose one unit. The edge crack is the line type. Net-like subepithelial capillary network patterns surround the gastric pits (dash arrow); B: Gastric body: The gastric pits are round (solid arrow). Honeycomb-like subepithelial capillary network patterns surround the gastric pits (dash arrows); C: Gastric antrum: The gastric pits are the line type (solid arrow). Coil-shaped subepithelial capillary network patterns surround the gastric pits (dash arrow).

The results are summarized in Table 1. The sensitivity and specificity of ME-NBI for predicting low grade gastric IN were 84.38% and 76.92% respectively. The overall accuracy of the ME-NBI diagnosis was 81% (95% CI: 69%-93%), and the kappa coefficient of agreement between the histopathology and the *in vivo* CLE imaging was 0.615. McNemar's test (using binomial distribution) indicated good agreement between the final histopathology and the CLE imaging ($P < 0.05$).

Comparative data in CLE + ME-NBI imaging and histopathology

Among 58 patients of gastric IN, 18 lesions with HIN and 32 lesions with LIN were diagnosed by ME-NBI with CLE. The results are summarized in Table 1. The sensitivity and specificity for predicting low grade gastric IN were 100% and 69.23% respectively. The overall accuracy of the ME-NBI diagnosis was 86.21% (95%CI: 73%-96%), and the kappa coefficient of agreement between the histopathology and the *in vivo* CLE imaging was 0.713. McNemar's test (using binomial distribution) indicated good agreement between final histopathology and CLE imaging ($P < 0.05$).

DISCUSSION

Endoscopy plays an important role in the early detection of GI tract neoplasms., It has been difficult to assess pre-malignant and early neoplastic lesions precisely using conventional white light or dye-based image enhanced endoscopy. NBI is an innovative optical technology that can increase the contrast of the precise morphological changes in the mucosal surface and clearly visualize microvascular structures. It dramatically improves the detec-

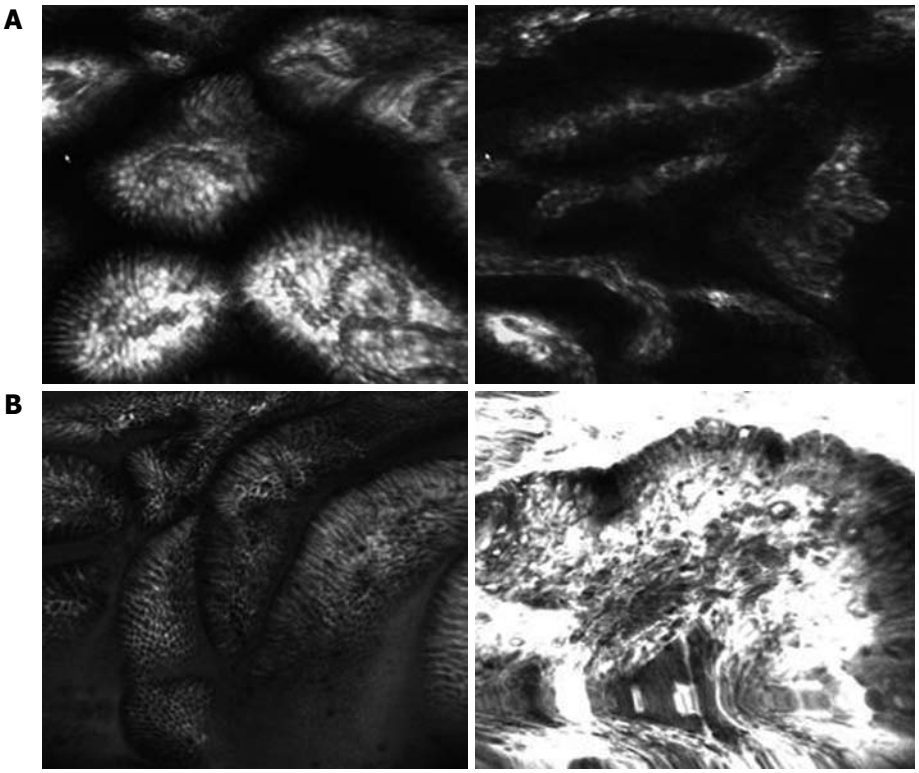


Figure 2 Confocal laser endomicroscopy images of low and high grade gastric intraepithelial neoplasia. A: Low grade gastric intraepithelial neoplasias show that in the different sizes of gastric pits, any capillary network are thickening and circuitous; B: High grade gastric intraepithelial neoplasias show the abnormal arrangement of gastric pits. The thickening capillary network and the increasing branch present a mass shape.

Table 1 Correlation between diagnosis made by confocal laser endomicroscopy, magnification endoscopy combined with narrow-band imaging, magnification endoscopy combined with narrow-band imaging + confocal laser endomicroscopy and histopathology in intraepithelial neoplasia

Histopathology (ESD)	LIN	HIN	Total
CLE			
LIN	29	4	33
HIN	3	22	25
ME-NBI			
LIN	27	6	33
HIN	5	20	25
ME-NBI + CLE			
LIN	32	8	40
HIN	0	18	18
Total	32	26	58

CLE: Confocal laser endomicroscopy; ME-NBI: Intraepithelial neoplasia by narrow-band imaging; LIN: Low grade intraepithelial neoplasia; HIN: High grade gastric intraepithelial neoplasia; ESD: Endoscopic submucosal dissection.

tion of pre-malignant and early neoplastic lesions, particularly in combination with magnification endoscopy. These features allow the better targeting of biopsies, and improved and more appropriate treatment, thereby contributing to optimal quality of life and patient survival. ME-NBI has allowed brand new possibilities for the endoscopic diagnosis of GI tract diseases.

Mucosal morphology includes microvascular architecture, and changes in the course of malignant transformation. There have been many studies on the vasculature in cancerous mucosa using magnification endoscopes or NBI. Sano *et al*[12] first reported the clinical utility of NBI in the GI tract in 2001. Nakayoshi *et al*[1] classified the MVP of superficial gastric cancers based on their magnifying NBI images into three groups: (1) fine network pattern; (2) corkscrew pattern; and (3) unclassified pattern. They also compared the endoscopic pattern with the histological findings. Fine network and corkscrew patterns are useful for identifying differentiated adenocarcinoma and undifferentiated adenocarcinoma, respectively. At the same time, they imply that magnifying NBI is also useful for identifying the lateral extension of superficial gastric cancer. In their reports, they studied the microvascular networks of 165 patients with depressed-type early gastric cancer lesions using a magnification endoscope with NBI and demonstrated that 66.1% of differentiated adenocarcinoma possessed fine networks, while 85.7% of undifferentiated adenocarcinoma had a corkscrew network[13]. In 2004, the clinical value of ME-NBI in the diagnosis and treatment of gastric neoplasia was been reported by Sumiyama's group. They came to the conclusion that it is important for the accurate assessment of the safety margin to ensure complete resection by endoscopic submucosal dissection or *en bloc* endoscopic mucosal resection for neoplastic lesions. In 2005, Yao *et al*[14,15] reported MVP is important for diagnosing early gastric carcinoma, as it provides 98.7% accuracy[16,17]. Then they implied the morphology of the white opaque substance (WOS) could be an alternative new optical sign for dis-

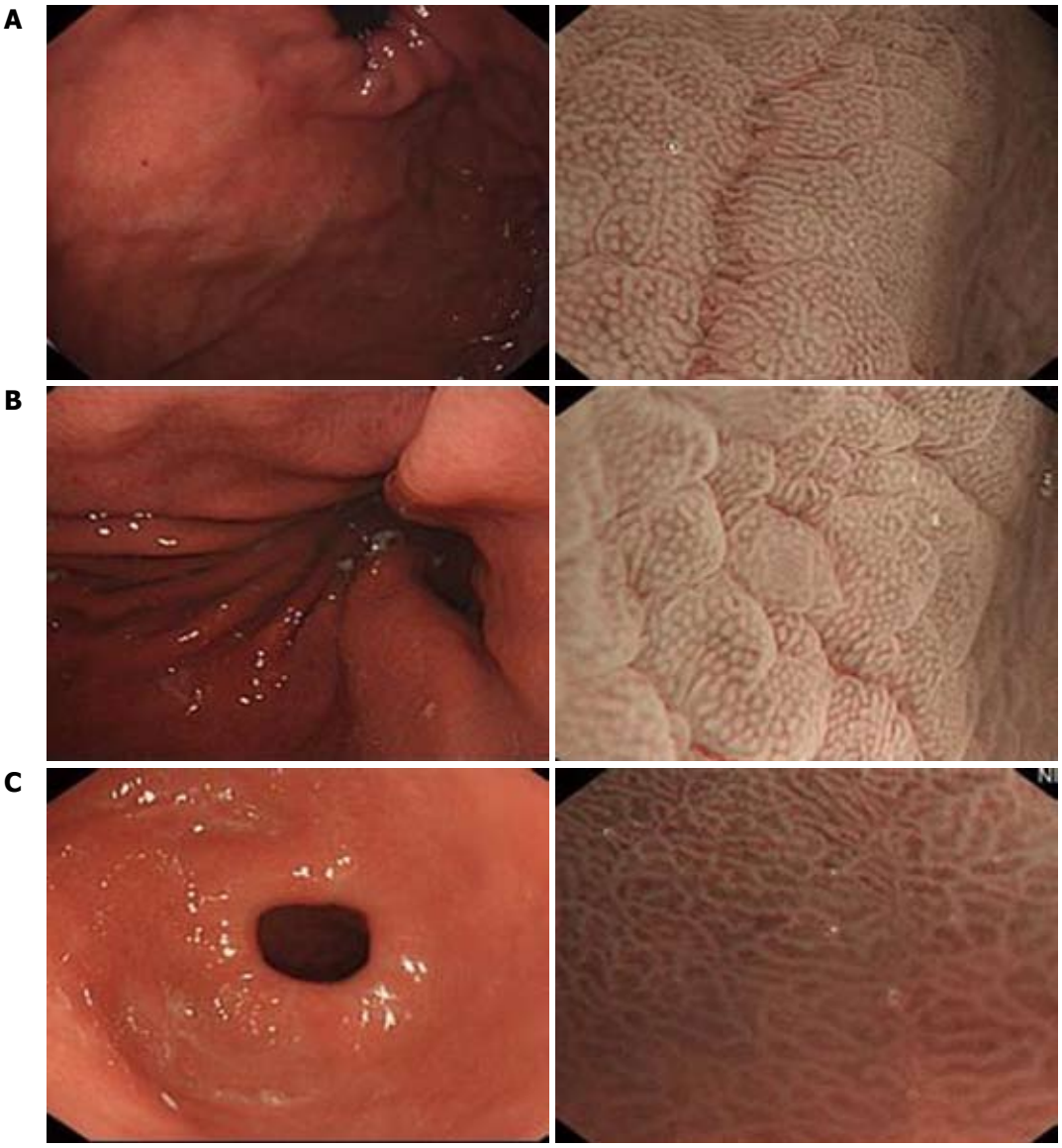


Figure 3 Routine endoscopy and intraepithelial neoplasia by narrow-band imaging images. A: Gastric fundus; B: Gastric body; C: Gastric antrum.

criminating adenoma from carcinoma.

In our studies, we found the WOS was one of the phenotypes in NBI magnification images, but not the main discrimination. However, the MSP and microvascular structures differ between LIN and HIN. LIN showed an irregular MSP and MVP with a clear demarcation line. The individual morphology of the crypt epithelium showed the different shapes (tubular, linear, curved or papillary). The vessels differ in shape (closed-loop, open-loop, tortuous or branched). Both the MSP and MVP have the irregular arrangement and variation in width and length. In contrast with LIN, HIN showed as flat because of a few areas without a network. The accuracy, sensitivity and specificity of ME-NBI for predicting low grade gastric IN were 81.03%, 84.38% and 76.92%, respectively.

Gastric mucosa IN is acknowledged as precancerous, with early diagnosis and prevention being important. To date, the final diagnosis still depends on histopathology. CLE has been developed which is an integration of a confocal laser microscope in the distal tip of a conventional videoendoscope. The components enable confocal microscopy in addition to standard videoendoscopy, and the new device can provide real-time, high magnification, cross-sectional images of the GI epithelium during routine endoscopy without the need for biopsy. Thusly, histopathology has been termed optical biopsy. This endoscopic tool allows for the observation of images magnified up to 1000-fold, which enables the visualization of mucosal microvascular architecture in units as small as the capillaries in the mucosal layer. Compared with other new optic techniques, the greatest advantage of CLE is that it can enables surface and subsurface imaging of living cells in the mucosa during ongoing endoscopy.

The first publication about an integrated confocal fluorescence microscope into the distal tip of a conventional colonoscope (Pentax EC 3830FK, Tokyo, Japan) was made by Kiesslich *et al*[18] in 2004, showing that *in vivo*

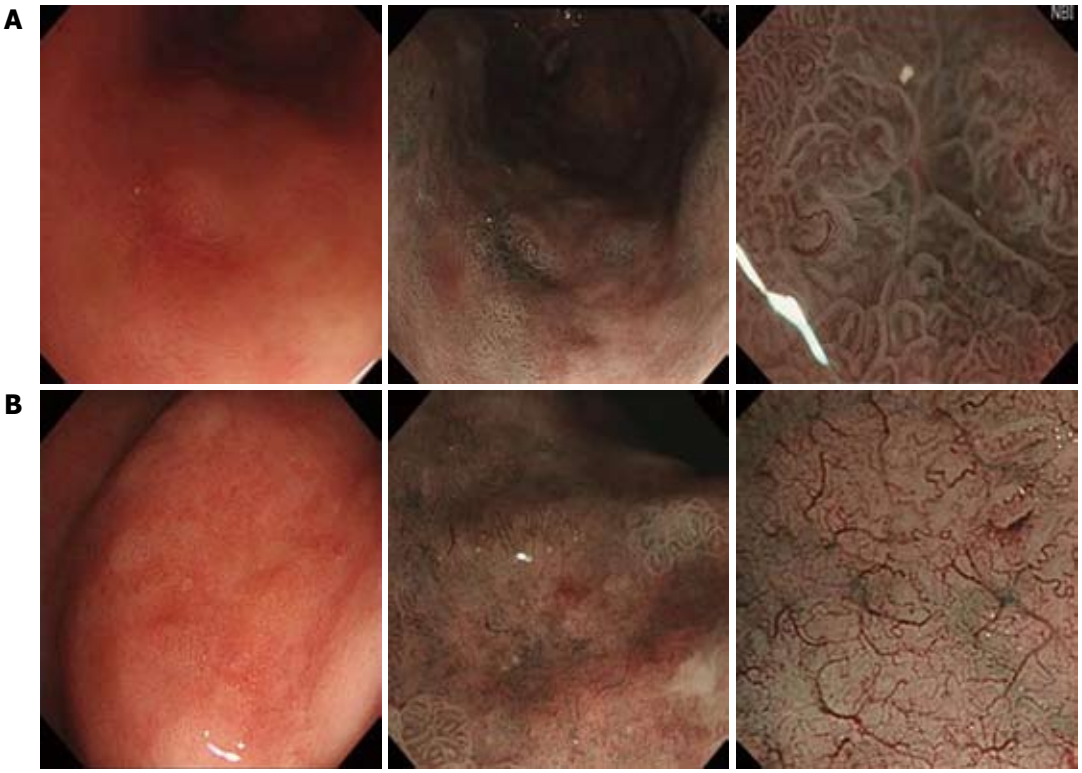


Figure 4 Routine endoscopy and narrow-band imaging images of low and high grade gastric intraepithelial neoplasia. A: Low grade intraepithelial neoplasias present an irregular surface and microvascular pattern with a clear demarcation line; B: High grade gastric intraepithelial neoplasias present the irregular surface pattern and microvascular pattern with a demarcation line. Few areas show as flat (absent microsurface pattern) with an obscured demarcation line.

microscopy at a subcellular resolution (0.7 μm) simultaneously displayed to white light endoscopy was possible and achieved high accuracy. This permitted immediate diagnosis of colorectal INs using fluorescein or acriflavine as contrast agents. Nuclear changes can best be observed using acriflavine as a topical contrast agent, as shown by Kakeji *et al*[19].

The endoscopic detection of early gastric cancer is challenging. Endomicroscopy allows immediate microscopic analysis of the gastric mucosa during ongoing endoscopy. Fluorescein-guided endomicroscopy is particularly good at demonstrating blood vessel architecture, which can be used to differentiate between non-neoplastic and neoplastic lesions. Kitabatake *et al*[20] showed the differences of mucosal vasculature according to the grade of tumor differentiation using CLE. The accuracy for the diagnosis of gastric cancer was above 90%. Liu *et al*[9] reported that comparison of regular and altered gastric mucosa facilitates the interpretation of the confocal images. Changes in tissue and microscopic blood vessel architecture, as well as changes in cell morphology, are valuable endomicroscopic criteria in defining neoplasias. Zhang *et al*[21] investigated the pattern and *in vivo* architecture of gastritis and gastric cancer on 132 consecutive patients. They classified gastric pit-pattern cellular architecture into seven types: normal mucosa with fundic glands mainly showed type A (round pits); corporal mucosa with histologic gastritis showed type B (non-continuous short rod-like); normal mucosa with pyloric glands mainly showed type C (continuous short rod-like); antral mucosa with histologic gastritis showed type D (elongated and tortuous branch-like); type E showed in intestinal metaplasia mucosa (the Goblet cells could be easily identified); and the corresponding values of the type G pattern (corkscrew-like and irregular in quality) for predicting gastric cancer were 90.0% and 99.4%.

Our study showed that the pit pattern and capillary network in the gastric mucosal layer was made visible using the confocal endomicroscope. In normal gastric antrum mucosa, the gastric pits are line type and the capillary network patterns showed a coil-shaped subepithelial capillary network pattern which surrounded the gastric pits. In the gastric body mucosa, the gastric pits are round and the capillary network patterns are characterized by a honeycomb-like network which was situated along or between the mucosal ridges. However, in the gastric fundus mucosa, the findings were incompatible with previous studies on gastric pit pattern and vascular network[21]; the superficial lamella gastric pits are round. Few pits compose one unit. The edge crack is the line type. Net-like subepithelial capillary network patterns surround the gastric pits.

When we studied gastric IN, we classified the mucosa into two types and observed the differences between them. LIN showed the different sizes and abnormal arrangement of gastric pits. The capillary network was thickening and circuitous. However, in the confocal images of HIN, the gastric pits presented strange shape. Some of them were unrecognized. A black tumor substance substituted the gastric patterns in some areas. The

thickening capillary network and the increasing branch present a mass shape. The accuracy, sensitivity and specificity of the CLE for predicting the low grade gastric IN were 87.93%, 90.63% and 84.62%, respectively. These findings do not conflict with the previous studies of Kitabatake *et al*[20].

The confocal images of normal gastric mucosa are similar to the NBI magnification images, but the MSP and MVP are more clearly visible by CLE than magnification endoscopy. The recent study of the diagnosis of the differentiation of gastric IN by the technique of CLE combined with ME-NBI has been lacking. Our study showed the accuracy, sensitivity and specificity of CLE combined with ME-NBI for predicting the low grade gastric IN were 86.21%, 100% and 69.23%, respectively. From the results, we know that the diagnostic accuracy and specificity for the differentiation of gastric IN by CLE are 87.93% and 84.62%, which is higher than the alternatives. CLE is the best technique for the diagnosis of the differentiation of gastric IN. However, the diagnostic sensitivity by CLE combined with ME-NBI is 100. This shows the consistency of the technique of CLE and NBI-ME for the diagnostic differentiation of gastric IN.

There are some limitations to CLE, in particular, the limited infiltration depth of the blue laser light. The depth of scanning is approximately 250 μm. Therefore the vessels in the submucosa were not visible by endomicroscopy. In addition, the collecting vein in gastric body mucosa was visible by magnifying NBI, but can not be discerned in confocal images. This is because CLE covers only a very small area while a collecting vein can be seen in about every 60 gastric pits. In conclusion, the CEM system provides precise and clear images of the microvascular architecture of gastric mucosa. There are distinct characteristics distinguishing normal and malignant superficial microvascular networks in the mucosal layer. Our findings suggest that changes in the MCV may indicate early endoscopically detectable signs of cancer, which might facilitate on-site diagnoses and enable better management.

When we analyzed the confocal images, we excluded some poor images. The main reason for this was the moving artifacts caused by GI motility, breath and heartbeats. In addition, excessive mucus often influenced laser scanning depth, which produced images without a clear microsurface or microvascular architecture. In clinical practice, both good and average images could be used for clinical analysis. The findings indicate that the images of normal mucosa were better than those of the cancerous mucosa. The reason for this is that the cancerous mucosa is not always flat. The confocal images of cancer were easily distinguishable from normal mucosa in our study.

We know the importance in using a contrast agent when performing CLE[19,22]. Fluorescein is inexpensive and non-mutagenic, and has been used in ophthalmology for decades. The subtle architecture of microvascular caliber vessel walls and even blood cells could be identified. The capillaries were much brighter than epithelial cells because of the flow of fluorescein in the vessels[20]. Nuclear changes can best be observed using acriflavine as a topical contrast agent. In our study, fluorescein was used as contrast agent, so that observation covered not only the surface structure, but also the deeper layer which displays the architecture of microvessels. However, we did not use the contrast agent acriflavine, so the nuclear changes could not be observed and analyzed in the confocal images.

We could not assess interobserver agreement about the mucosal patterns seen in magnification NBI and CLE in this study. Future studies could determine whether our results can be reproduced by using two blinded observers. Our study needs further longitudinal investigation. The other limitation of this study was the small number of cases. Further prospective blinded studies with a sufficient number of patients are therefore important.

In conclusion, these endoscopic techniques of magnifying endoscopy with NBI and CLE that can provide valuable information regarding normal gastric mucosa and should be used for evaluating the grade of gastric IN.

ACKNOWLEDGMENTS

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COMMENTS

Background

Diagnosis and localization of early gastric cancer is problematic because of the lack of any endoscopic characteristics, even with multiple random biopsies. In recent years, endoscopic image quality has improved. Magnifying endoscopy combined with narrow-band imaging (ME-NBI) has been used to achieve endoscopic pathology in gastric disease. Confocal laser endomicroscopy (CLE) is a newly-developed technology that has enabled endoscopists to collect real-time *in vivo* histological images or "virtual biopsies" of the gastrointestinal (GI) mucosa during endoscopy, and has stimulated significant interest in the application of this technique in clinical gastroenterology. The following attempts to concisely and accurately summarize the related background of the article and to enable the readers to gain some basic knowledge relevant to the article, thus helping them better understand its significance.

Research frontiers

Any prognosis of GI cancer is closely related to the stage of the disease at diagnosis. Endoscopic submucosal dissection (ESD) and *en bloc* endoscopic mucosal resection (EMR) have been performed as curative treatments for many early-stage GI lesions in recent years. However, before engaging in endoscopic therapy, an accurate diagnosis is a precondition affecting the complete cure of the underlying malignancy or carcinoma *in situ*. For the past few years, many new types of endoscopic techniques, including ME-NBI and CLE, have emerged in many countries since these methods provide a strong indication of early lesions and are very useful in determining treatment options before ESD or EMR.

Innovations and breakthroughs

This study showed that the pit pattern and capillary network in the gastric mucosal layer was made visible using the confocal endomicroscopy. In normal gastric antrum mucosa, the gastric pits are of the line type and the capillary network patterns showed a coil-shaped subepithelial capillary network pattern which surrounded the gastric pits. In the gastric body mucosa, the gastric pits are round and the capillary network patterns are characterized by a honeycomb-like network which was situated along or between the mucosal ridges.

Applications

The diagnostic accuracy and specificity for the differentiation of gastric IN by CLE are 87.93% and 84.62%, which is higher than the alternatives. CLE is the best technique for the diagnosis of the differentiation of gastric intraepithelial neoplasia (IN). However, the diagnostic sensitivity of CLE combined with ME-NBI is 100%, which showed the consistency of CLE and NBI-ME for the diagnostic differentiation of gastric IN.

Terminology

Gastric carcinoma is the second commonest cause of cancer deaths worldwide. Early detection and diagnosis of gastric cancer in the stomach is important for improving patient prognosis. CLE is a novel endoscopic modality that allows subsurface analysis of the gastric mucosa during ongoing endoscopy. ME-NBI is more accurate in the diagnosis of gastric cancer when the diagnostic triad of the disappearance of fine mucosal structure, microvascular dilation, and heterogeneity is used. Several studies have reported that this technique is of value in the diagnosis of premalignant lesions in the GI tract. Patients underwent an examination with CLE and magnifying NBI. Obtained image resolution was evaluated, and the morphology, pit patterns and blood capillary forms of lesions were analyzed. The results indicate the feasibility of these two techniques for the efficacious diagnostic classification of gastric INs.

Peer review

For the study of gastric INs, the authors classified the mucosa into two types and observed the differences between them. Low grade gastric intraepithelial neoplasia (LIN) showed different sizes and an abnormal arrange of gastric pits. In the confocal images of high grade gastric intraepithelial neoplasias (HIN), the gastric pits presented strange shapes. The results showed that white opaque substance is one of the phenotypes in NBI magnification images, but not the main discrimination. The microsurface pattern and microvascular structures differ between LIN and HIN. LIN showed the irregular microsurface and microvascular pattern with a clear demarcation line. The study also showed that the pit pattern and capillary network in the gastric mucosal layer was made visible using a confocal endomicroscope. The method for CLE, ME-NBI is clearly stated.

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BRIEF ARTICLE

Hyperthermia inhibits hypoxia-induced epithelial-mesenchymal transition in HepG2 hepatocellular carcinoma cells

Guang-Jin Yuan, Qian-Wen Li, Shun-Lin Shan, Wu-Ming Wang, Sen Jiang, Xi-Ming Xu

Guang-Jin Yuan, Qian-Wen Li, Shun-Lin Shan, Wu-Ming Wang, Sen Jiang, Cancer Center, the 82nd Hospital of the Chinese People's Liberation Army, Huai'an 223001, Jiangsu Province, China
Xi-Ming Xu, Cancer Center, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China
Author contributions: Yuan GJ and Li QW designed the study; Wang WM and Jiang S performed the majority of experiments; Shan SL analyzed the data; and Yuan GJ and Xu XM wrote the paper.
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Correspondence to: Xi-Ming Xu, Professor, PhD, Cancer Center, Renmin Hospital of Wuhan University, No. 9 Ziyang road, Wuhan 430060, Hubei Province, China. whuxxm@yahoo.com
Telephone: +86-27-88041911 Fax: +86-27-88042292
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Abstract

AIM: To investigate the effect of hyperthermia on hypoxia-induced epithelial-mesenchymal transition (EMT) in HepG2 hepatocellular carcinoma (HCC) cells, and its mechanism.

METHODS: Cells were treated with hyperthermia at 43 ℃ for 0.5 h, followed by incubation under hypoxic or normoxic conditions for 72 h. Cell morphology was observed. Expressions of E-cadherin and vimentin were determined by immunofluorescence assay or Western blot. The protein and mRNA expressions of Snail were also determined by Western blot and reverse transcription-polymerase chain reaction. Cell migratory capacity was evaluated.

RESULTS: Hypoxia induced EMT in HepG2 cells, which was evidenced by morphological, molecular and functional changes, including the formation of a spindle shape and the loss of cell contact. The expression of E-cadherin was decreased but the expression of vimentin was increased; also, the migratory capability was increased by 2.2 ± 0.20-fold as compared with normoxia. However, those effects were inhibited by hyperthermia pretreatment. Furthermore, protein synthesis and mRNA expression of Snail in the cells were enhanced by hypoxia as compared with normoxia, and also significantly inhibited by hyperthermia pretreatment.

CONCLUSION: Hyperthermia may inhibit hypoxia-induced EMT in HepG2 HCC cells, and the mechanism may involve inhibition of induced expression of Snail.

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Key words: Hyperthermia; Hypoxia; Epithelial-mesenchymal transition; Hepatocellular carcinoma; Snail

Peer reviewer: Dr. Xian-Ming Chen, Department of Medical Microbiology and Immunology, Creighton University Medical Center, 2500 California Plaza, Omaha, NE 68178, United States

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide, and ranks third in the leading causes of cancer mortality[1]. There are approximately 560 000 new cases diagnosed each year, and around 550 000 deaths occur mostly in developing countries[2]. However, the choices for its treatment, especially for advanced HCC, is limited, and the prognosis is poor.

Hyperthermia is a physical treatment with fewer side effects than chemotherapy and radiotherapy. Numerous phase Ⅱ/Ⅲ studies have shown that hyperthermia is feasible and effective especially if combined with chemotherapy or radiotherapy in a variety of solid tumors (e.g., melanoma, breast cancer, HCC)[3-5]. It is reported that local hyperthermia can inhibit metastasis in an animal model[6]; however, the exact mechanisms remain unclear. Epithelial-mesenchymal transition (EMT) is a key step during cancer invasion and metastasis[7], and has been associated with cancer stem cells and resistance to chemotherapy and radiotherapy[8-10]. In the present study, we investigated the effect of hyperthermia on hypoxia-induced EMT in HepG2 HCC cells, and its underlying mechanism.

MATERIALS AND METHODS

Cell line culture

The human HCC cell line HepG2 (Wuhan University Cell Center) was cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/mL streptomycin, and 100 U/mL penicillin in a humidified 5% CO_2 atmosphere at 37 ℃.

Cell hyperthermia and hypoxia treatment

Water-bath warming was performed and the temperature was controlled at 43 ℃. The cell culture bottles were packaged and put into the water at 43 ℃ for 0.5 h. Then the culture bottles were returned to the cell-incubator immediately and incubated at 37 ℃ for 24 h. Thereafter, the cells were incubated under hypoxic (1% O_2, 5% CO_2, 37 ℃) or normoxic (21% O_2, 5% CO_2, 37 ℃) conditions for 72 h.

Immunofluorescence detection of E-cadherin

After hyperthermia, cells were plated on sterile coverslips. After 24 h, the cells were treated with hypoxia as mentioned above. Then, the cells were fixed in 4% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 10% goat serum for 60 min at room temperature. Thereafter, cells were incubated with antibodies specific for goat E-cadherin (SC-31020, diluted 1:50) at 4 ℃ overnight. After washes with phosphate-buffered saline (PBS) 3 times, the cells were incubated with Alexa Fluor® 488 mouse anti-goat IgG (H+L) (A11078, diluted 1:200) for 60 min at room temperature. The expression of E-cadherin was detected under the fluorescence microscope.

Western blot analysis

Protein lysates were prepared using total protein extraction kit (ProMab, SJ-200501), and stored at -20 ℃ until assay. The protein concentrations were determined with the Bradford method. The equivalent aliquots of protein were separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis, then transferred onto a nitrocellulose filter. The filters were blocked with 5% nonfat dry milk in PBS for 2 h at 37 ℃. After washing with PBST (PBS with Tween 20), they were incubated at 4 ℃ overnight with goat E-cadherin (SANTA, SC-31020, diluted 1:400), mouse vimentin (ProMab, 20301, diluted 1:1000), mouse Snail (CST, 3895, diluted 1:500) or mouse beta-actin (ProMab, 20270, diluted 1:1000) antibodies. Following 4-time PBST washing, the filters were incubated with secondary antibody of horseradish peroxidase-conjugated rabbit anti-goat IgG (diluted 1:4000, for E-cadherin) or goat anti-mouse IgG (diluted 1:5000, for vimentin, Snail and β-actin) for 1 h at room temperature. The immunoreactive proteins were detected using an enhanced chemiluminescent detection system.

Reverse transcription-polymerase chain reaction analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) as suggested by the supplier. One micrograms of total RNA were reverse-transcribed. The sequences for forward and reverse primers were 5'-TCAACTGCAAATACTGCAAC-3' and 5'-CTTGACATCTGAGTGGGTCT-3' (Snail), and 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). The sizes of their amplified fragments were 242 and 450 bps, respectively. Amplification was performed with 40 cycles with initial incubation at 95 ℃ for 5 min and final extension at 72 ℃ for 5 min. Each cycle consisted of denaturation for 30 s at 94 ℃, annealing for 30 s at 54 ℃ and extension for 30 s at 72 ℃. Following PCR, 10 μL samples of amplified products were resolved by electrophoresis in 1% agarose gel, then stained with ethidium bromide. The levels of Snail expression were semiquantitatively evaluated using a Kodak ultraviolet imaging system and an image analysis system (Vilber Lourmat, France), and normalized to GAPDH.

Cell migration assay

Cell migration was assessed using 6.5-mm Costar Transwell chambers with 8 μm pores (Corning). Cells were trypsinized and resuspended in serum-free medium containing 0.5% bovine serum albumin. Cells (5×10^4) were plated in the upper chamber in the above serum-free medium, and 1 mL serum-containing standard medium was placed in the lower chamber as a chemoattractant. The system was incubated in 1.0% or 21% O_2 for 24 h. After incubation, the nonmigrating cells were removed from the upper surface of the Transwell membrane by a cotton swab, and the membrane was fixed with 95% ethanol for 15-20 min before staining with hematoxylin and eosin. Cells that migrated through the membrane to the lower surface were counted under light microscopy.

Statistical analysis

Data were pooled from at least three independent experiments, and presented as mean ± SD unless otherwise indicated. Differences between groups were analyzed using one-way analysis of variance. All the statistical analyses were performed with SPSS13.0. $P < 0.05$ was considered statistically significant.

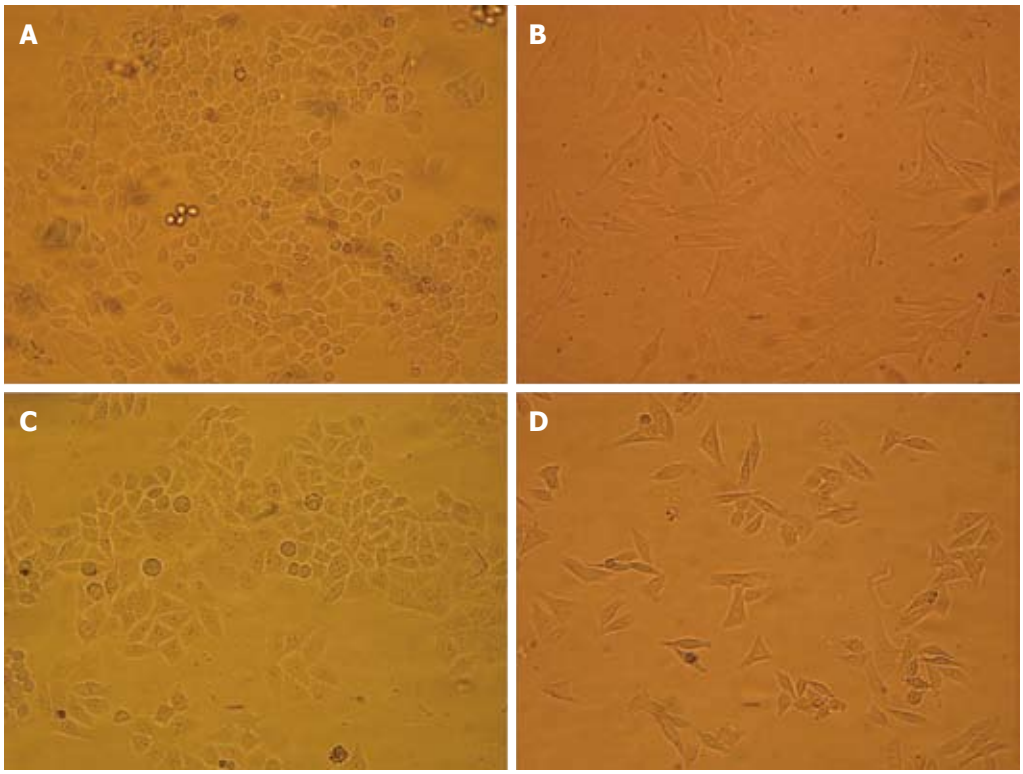


Figure 1 Morphologic changes after hyperthermia or hypoxia treatment in HepG2 cells. A: Cells were cutured in 21% O_2 for 72 h; B: Cells were cultured in 1.0% O_2 for 72 h; C: Cells were treated with hyperthermia at 43 ℃ for 0.5 h, followed by treatment with normoxia; D: Cells were treated with hyperthermia at 43 ℃ for 0.5 h, followed by treatment with hypoxia.

RESULTS

Effects of hyperthermia and hypoxia on cell morphology

Folding and corrugation of the cell membrane, and a number of vacuoles in the cytoplasm were observed after hyperthermia at 43 ℃ for 0.5 h. The cells cultured in 1.0% O_2 for 72 h showed a spindle shape and lost cell contact. However, these features were partially inhibited by pretreatment with hyperthermia (Figure 1).

Effects of hyperthermia and hypoxia on expressions of E-cadherin and vimentin

Expressions of E-cadherin were determined by immunofluorescence assay and Western blot. As shown in Figure 2, E-cadherin immunofluorescence staining intensity was significantly decreased in cells after incubation in 1.0% O_2 for 72 h as compared with cells cultured in normoxic conditions. However, hyperthermia pretreatment significantly blunted the above effects caused by hypoxia. As compared with untreated cells, hyperthermia alone moderately increased the immunofluorescence intensity of E-cadherin staining.

Consistent with the results obtained in immunofluorescence assays, Western blot analysis also showed that, under hypoxia, the expression of E-cadherin was significantly reduced, but the expression of vimentin was substantially increased when compared with normoxia. These effects were blunted after hyperthermia pretreatment (Figure 3). Hyperthermia alone also moderately increased the expression of E-cadherin but decreased the expression of vimentin compared with untreated cells.

Effects of hyperthermia and hypoxia on the protein and mRNA expressions of Snail

The protein and mRNA expressions of Snail were determined by Western blot and reverse transcription-polymerase chain reaction analysis, respectively. Similar to the expression of vimentin, the protein expression of Snail was significantly increased under hypoxia as compared with normoxia, and hyperthermia pretreatment inhibited the effect induced by hypoxia (Figure 4A). Hyperthermia alone moderately decreased the protein expression of Snail compared with untreated cells.

Consistent with protein expression, Snail mRNA expression was induced under hypoxia, but its induction was inhibited by hyperthermia pretreatment (Figure 4B). Hyperthermia alone also moderately decreased the mRNA expression of Snail compared with untreated cells.

Effects of hyperthermia and hypoxia on cell migration

Transwell chamber assays were performed to investigate the migratory capability of HepG2 cells treated with hyperthermia and hypoxia. Under hypoxia, cell migration was increased by 2.2 ± 0.20-fold as compared with normoxia ($P < 0.01$), which was attenuated to 1.4 ± 0.25-fold by hyperthermia pretreatment (Figure 5). Hyperthermia alone moderately decreased the cell migration compared with untreated cells.

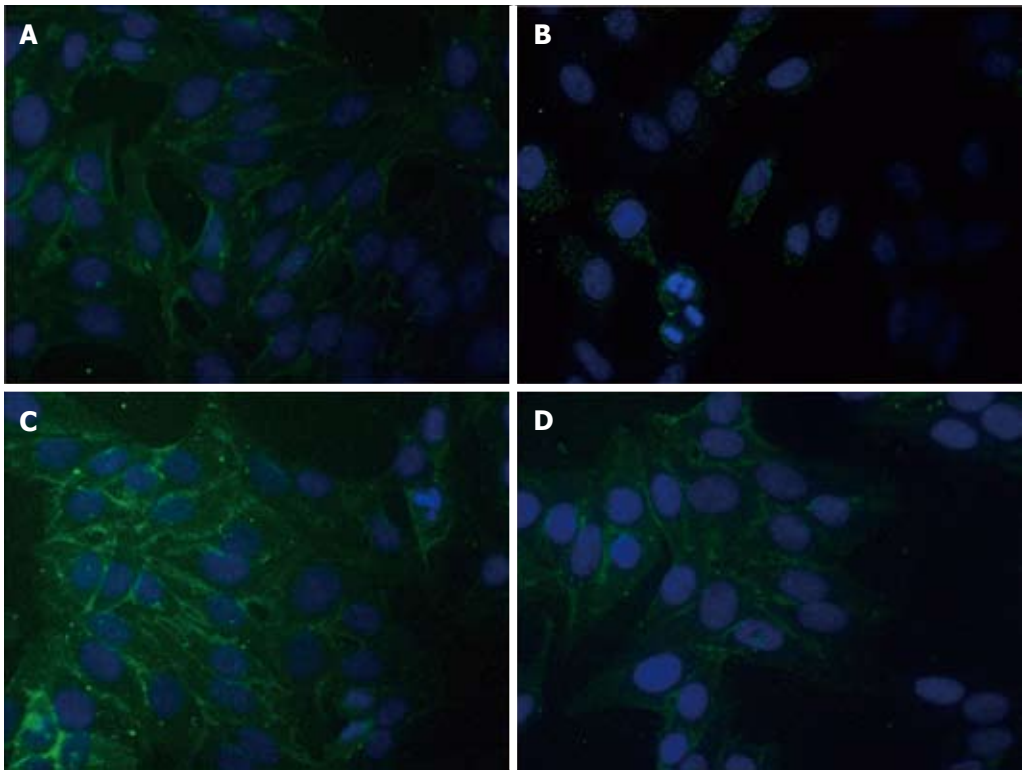


Figure 2 Immunofluorescence assay of E-cadherin in HepG2 cells. A: Untreated cells; B: Hypoxia-treated cells; C: Hyperthermia-treated cells; D: Hyperthermia + hypoxia-treated cells.

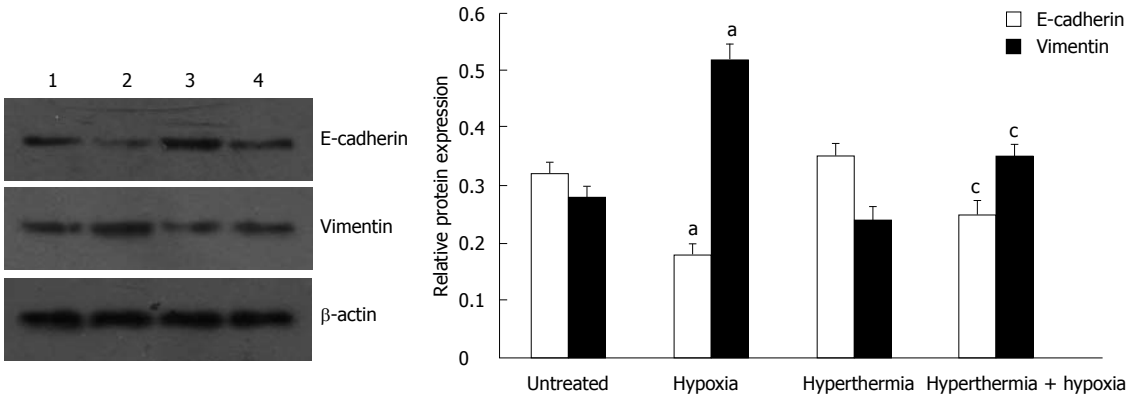


Figure 3 Western blot analysis of E-cadherin and vimentin expression in HepG2 cells. Lanes 1, 2, 3 and 4 represent untreated cells, hypoxia-treated cells, hyperthermia-treated cells and hyperthermia + hypoxia-treated cells, respectively. The bar graphs show the relative expression levels of indicated proteins after normalization to β-actin. $^{a}P < 0.05$ *vs* untreated cells; $^{c}P < 0.05$ *vs* hypoxia-treated cells.

DISCUSSION

Hypoxia is a common hallmark of solid cancers, including liver cancer, and is associated with treatment failure and poor prognosis[11,12]. Previous studies have shown that it can induce both chemo- and radio-resistance, and also promotes cell migration, invasion and metastasis[11-13]. EMT, originally identified as a process vital for morphogenesis during embryonic development, is now recognized as a key step during cancer invasion and metastasis[7], and has been associated with cancer stem cells and resistance to chemotherapy and radiotherapy[8-10]. EMT is characterized by the loss of epithelial cell markers such as adherens protein E-cadherin, the acquisition of mesenchymal markers such as vimentin and fibronectin, and an increase in cell motility[9]. Several studies have shown that hypoxic conditions can induce EMT in cancer cells, the mechanisms of which may involve reactive oxygen species generated during hypoxia, hypoxia-inducible factor-1α and phosphoinositide-3-kinase/Akt signaling[14-16]. Consistent with other studies, the present study showed that hypoxia induced morphological (spindle shape and loss of cell contact), molecular (decreased E-cadherin but increased vimentin expressions) and functional (increased cell migration) changes in HepG2 HCC cells, all of which are in line with EMT.

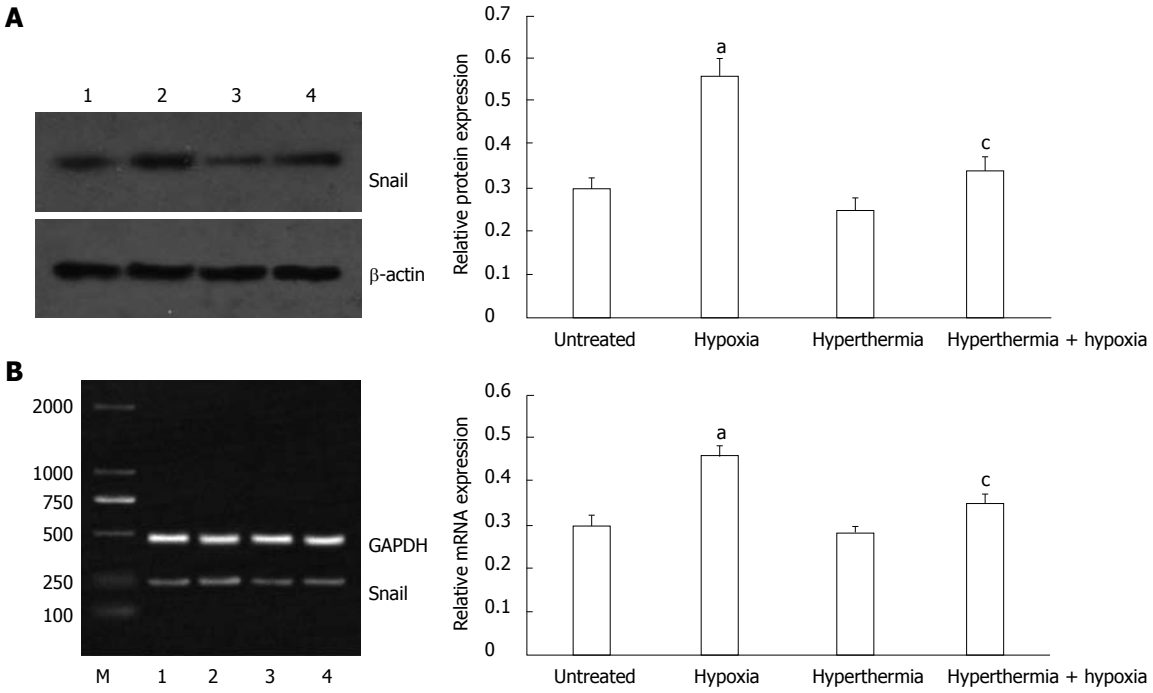


Figure 4 Western blot and reverse transcription-polymerase chain reaction analysis of Snail expression in HepG2 cells. A: Western blot: Lanes 1, 2, 3 and 4 represent untreated cells, hypoxia-treated cells, hyperthermia-treated cells and hyperthermia + hypoxia-treated cells, respectively. The bar graphs show the relative expression levels of Snail after normalization to β-actin; B: Reverse transcription-polymerase chain reaction: The representation of the lanes was the same as in (A); the bar graphs show the relative expression levels of Snail normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). $^{a}P < 0.05$ *vs* untreated cells; $^{c}P < 0.05$ *vs* hypoxia-treated cells.

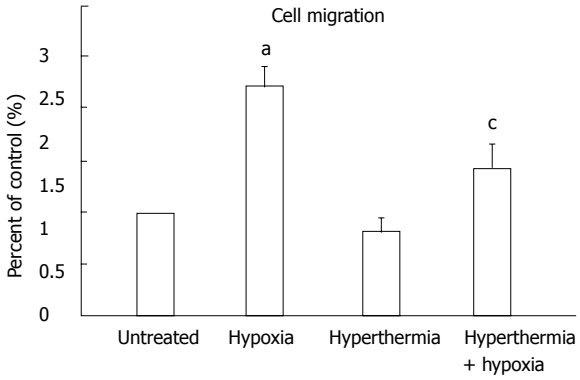


Figure 5 Migration assay of HepG2 cells treated with hyperthermia or hypoxia. Quantification of the cell migration results from three experiments: each bar (hypoxia-, hyperthermia-, hyperthermia + hypoxia-treated cells) represents the relative levels observed in untreated cells (control). $^{a}P < 0.05$ *vs* untreated cells; $^{c}P < 0.05$ *vs* hypoxia-treated cells.

In recent years, hyperthermia has emerged as an effective approach for cancers, with fewer side effects than chemotherapy and radiotherapy. Clinical studies have shown that hyperthermia, usually classified as local, regional and whole body, is a tolerable and clinical practical adjunctive therapy for the patients with advanced malignant tumors[3-5]. Previous reports have demonstrated that local hyperthermia can inhibit tumor metastasis in animal model[6]. Furthermore, the expressions of some genes associated with metastasis such as vascular endothelial growth factor, membrane type 1-matrix metalloproteinase, were shown to be suppressed by heating[17-19]. In the present study, we found that hyperthermia can inhibit hypoxia-induced EMT, as hyperthermia before hypoxia exposure significantly blunted the inhibition of E-cadherin, the induction of vimentin expressions, and the increase of cell migration triggered by hypoxia in HepG2 HCC cells. These findings suggested that hyperthermia may alter the metastatic potential of cancer cells and inhibit tumor metastasis.

A number of transcription factors have been found to play an important role in EMT, including zinc finger homologs (e.g., Snail), basic helix-loop-helix transcription factors (e.g., Twist) and ZEB family members (ZEB1, ZEB2/SIP1)[9]. Snail has been found to be expressed in numerous human tumors, including gastric and hepatic carcinoma[20-22], and has been described as a direct repressor of E-cadherin both *in vitro* and *in vivo* through binding to E-box elements in the E-cadherin promoter, repressing its expression and producing changes in cell phenotype consistent with EMT[23,24]. Our experiments showed that hypoxia induced mRNA and protein expressions of Snail in HepG2 HCC cells, which may be involved in the mechanism of hypoxia-induced EMT. However, the induction of Snail expressions by hypoxia was inhibited by hyperthermia pretreatment. Therefore, the mechanism by which hyperthermia inhibits hypoxia-induced EMT may involve the reduction in Snail expression.

Taken together, the present study demonstrated that hyperthermia inhibits hypoxia-induced EMT in HepG2 HCC cells, and the mechanism may be associated with the reduction of mRNA and protein expressions of Snail. This study provides further evidence that hyperthermia can inhibit tumor metastasis.

COMMENTS

Background

Epithelial-mesenchymal transition (EMT) has been recognized as a key step during cancer invasion and metastasis, and may be induced by environmental stresses such as hypoxia, reactive oxygen species, and growth factors such as transforming growth factor β1. Hypoxia is a common phenomenon in solid tumors, and hypoxic conditions are known to induce EMT in cancer cells. Hyperthermia is a potent approach for cancers with fewer side effects compared with chemotherapy and radiotherapy. Recent reports have shown that local hyperthermia can inhibit tumor metastasis in an animal model, and the mechanisms are associated with vascular endothelial growth factor, membrane type 1-matrix metalloproteinase. However, the effect of hyperthermia on EMT of cancer cells is unknown.

Research frontiers

EMT plays an essential role in tumor progression and metastasis, and has been associated with cancer stem cells and resistance to chemotherapy and radiotherapy. To reverse EMT or to target EMT related pathways is a promising novel approach to overcome the chemoresistance and radioresistance for cancer treatment, and to help prevent tumor metastasis.

Innovations and breakthroughs

Hyperthermia can inhibit EMT induction in cancer cells, suggesting that hyperthermia may alter the properties of metastatic potential of cancer cells and inhibit tumor metastasis. Furthermore, the mechanism of inhibition is associated with inhibition of expression of the transcription factor Snail.

Applications

The results of the study provide a novel theoretical basis for hyperthermia in the treatment of malignant tumors, especially combined with chemotherapy or radiotherapy.

Terminology

EMT is a physiological and pathological process in which epithelial cells acquire the motile and invasive characteristics of mesenchymal cells. EMT is characterized by the loss of epithelial cell markers such as E-cadherin, the acquisition of mesenchymal markers such as vimentin and fibronectin, and an increase of cell motility. EMT has been recognized as a key step during embryonic morphogenesis, heart development, chronic degenerative fibrosis, and cancer metastasis.

Peer review

The paper described the effect of hyperthermia on EMT in HepG2 hepatocellular carcinoma cells, and its associated mechanism. The authors concluded that hyperthermia may inhibit EMT induction in cancer cells and the mechanism is associated with inhibition of expression of the transcription factor Snail. The results are interesting, but the mechanism needs to be further studied, especially employing Snail knockdown technique.

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CASE REPORT

Angioedema associated with Crohn's disease: Response to biologics

Flavio Habal, Vivian Huang

Flavio Habal, Vivian Huang, Department of Gastroenterology, University Health Network, University of Toronto, Toronto ON M5G 2C4, Canada
Author contributions: Habal F managed the patients and provided case data and photographs, as well as editing; Huang V completed the literature review and hypothesis generation, and wrote the paper.
Correspondence to: Flavio Habal, MD, Department of Gastroenterology University Health Network, University of Toronto, 200 Elizabeth Street, Toronto ON M5G 2C4, Canada. flavio.habal@uhn.ca
Telephone: +1-416-3405023 Fax: +1-416-5955251
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Peer reviewer: Bruno Bonaz, MD, PhD, Professor, Department of Gastroenterology, Grenoble University, 38000 Grenoble, France

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Abstract

A 46-year-old female patient with terminal ileum Crohn's disease and ankylosing spondylitis presented with recurrent angioedema and urticaria. Investigations ruled out hereditary angioedema, and environmental or food allergen triggers. She was diagnosed with chronic idiopathic urticaria with angioedema, and was treated with a trial of intravenous immunoglobulin immunotherapy, danazol, prednisone and hydroxyzine. Due to ongoing bowel and arthritic complaints, she was started on infliximab infusions and within 2 treatments, she had complete resolution of the angioedema and urticaria, as well as of the bowel and arthritic symptoms. Unfortunately she developed allergic reactions to the infliximab and was switched to another anti-tumor necrosis factor (TNF)-α agent, adalimumab. Since then, she has had no further angioedema or urticaria, and her Crohn's disease has been quiescent. This is the first known case report of chronic idiopathic urticaria with angioedema coexistent with Crohn's disease that was successfully treated with anti-TNF-α agents.

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INTRODUCTION

Studies have shown that there is a strong autoimmune involvement in the pathophysiology of urticaria and angioedema. There are increases in pro-inflammatory cytokines, such as interleukin (IL)-1 β, IL-12p70, tumor necrosis factor (TNF)-α, IL-6, IL-10 and IL-17, in chronic idiopathic urticaria. Crohn's disease also has autoimmune involvement, and there is evidence for an altered cytokine milieu leading to mucosal inflammation. Recent studies have shown that T-cell production of certain cytokines play a strong role. One common thread in the pathophysiology of chronic idiopathic urticaria and Crohn's disease is the derangement in cytokine levels, in particular, IL-17 and TNF-α. The IL-17 cytokines are T-cell derived cytokines that stimulate various cells to secrete cytokines and chemokines, and therefore play an important role in many autoimmune diseases. It has been shown that IL-17A positive cells are increased in the inflamed mucosa of inflammatory bowel disease (IBD) patients, and IL-17F mRNA expression is elevated in the mucosa of Crohn's disease patients. Infliximab and adalimumab are anti-TNF-α agents that block the inflammatory cascade. Given the similarity in cytokine derangements found in

chronic idiopathic urticaria and in Crohn's disease, medications that target these cytokines, such as anti-TNF-α agents should be effective in both conditions.

This case brings to attention the common pathophysiology between autoimmune diseases, and the need for further research looking into the changes in the cytokine milieu as potential targets for treatment.

CASE REPORT

A 46-year-old female with terminal ileum Crohn's disease and ankylosing spondylitis presented to our Gastroenterology clinic with recurrent angioedema and urticaria. Her Crohn's disease was diagnosed in 1993 when she began having abdominal pain and diarrhea. A small bowel follow through revealed terminal ileum involvement, and she was started on Pentasa at that time. She remained well on Pentasa and for unclear reasons discontinued the medication in 2001. She was diagnosed with ankylosing spondylitis in 2004 just prior to presentation. On examination, she had angioedema and urticaria involving the tongue, lips, eyes, face, feet, hands and genital areas (Figure 1). Laboratory investigations revealed white blood cell 1.0 (normal, range: 4.0×10^9 - 11.0×10^9/L), neutrophils 10.0 (normal, range: 2.0×10^9 - 7.50×10^9/L), erythrocyte sedimentation rate 68 (normal, range: 0-20 mm/h). She was restarted on an aminosalicylate, Pentasa, for treatment of active Crohn's disease with the concern that the angioedema may be related to the activity of the Crohn's disease. A subsequent colonoscopy revealed aphthous ulceration in the terminal ileum. Biopsies of the ileocecal valve and terminal ileum showed mild acute on chronic inflammation with no granulomas and no dysplasia.

After restarting the Pentasa, her urticaria and angioedema subsided for 3 mo. Unfortunately her symptoms then recurred. The episodes became more frequent, and she required Prednisone and Atarax to control her symptoms. An allergist performed skin testing which revealed environmental allergies but no significant food allergies. Further investigations revealed an elevated IgM (3.02, range: 0.2-2.2), elevated IgE (1120) without eosinophilia, normal C3 (1.19), and normal C4. There was a question of hereditary angioedema, however she had normal C1 esterase inhibitor levels (313). Based on these results, she was diagnosed with chronic idiopathic recurrent urticaria with angioedema.

She was treated with a trial of intravenous immunoglobulin immunotherapy, but developed aseptic meningitis, and was instead treated with repeated courses of prednisone. A trial of Danazol did not alleviate her symptoms and she continued to be dependent on Prednisone and Atarax.

In 2007, because of her ongoing bowel, arthritis, and urticaria/angioedema symptoms, she was started on intravenous infliximab infusions (5 mg/kg) and within two treatments (weeks 0 and 2); she improved with resolution of the angioedema and urticaria, as well as of the bowel and arthritic symptoms. Unfortunately, while receiving her thirteenth infusion, she developed allergic reactions to the Infliximab. She was switched to another anti-TNF-α agent, adalimumab (160 mg subcut week 0, 80 mg week 2, 40 mg week 4 then 40 mg 2 wk). We have continued to follow her and since then, she has had no further angioedema or urticaria, and her Crohn's disease has been quiescent. This is the first known case report of chronic idiopathic urticaria with angioedema coexistent with Crohn's disease that was successfully treated with anti-TNF-α agents.

DISCUSSION

Urticaria refers to swelling of the superficial skin layer, and can be acute or chronic (present for at least 6 wk). When no external cause can be found for chronic urticaria, the patient is referred to as having "chronic idiopathic urticaria"[1]. Urticaria can occur alone (approximately 40% of patients), or in combination with angioedema (approximately 50% of patients)[1]. Angioedema refers to non-pruritic, non-pitting areas of swelling or cutaneous and mucosal tissues, affecting the deep dermal and subcutaneous/submucosal skin layers[1,2]. Angioedema can be further categorized into hereditary, acquired, associated with allergic reactions, and idiopathic. Degranulation and histamine release by mast cells and basophils leads to the erythema and edema typical of urticaria and angioedema.

Studies have shown that there is a strong autoimmune involvement in the pathophysiology of urticaria and angioedema. These conditions have been associated with other autoimmune diseases such as autoimmune thyroid disease and celiac disease[1,2]. Chronic urticaria and angioedema have been attributed to reactions to food, drugs, environmental antigens, and *Helicobacter pylori* infection, but the etiology for a majority of patients remains unknown. However, even for these "idiopathic" chronic urticaria patients, it is believed that the underlying mechanism is an autoimmune phenomenon[1,3,4].

Up to 30%-50% of patients with chronic urticaria have autoantibodies to the α-chain of the high affinity receptor for IgE (FceRIa); it is thought that these autoantibodies cross-link the IgE receptors, therefore activating the infiltrating basophils and skin mast cells, leading to histamine release[1,3,4].

In addition, other circulating mediators may play a role in activation and histamine release studies have shown increases in pro-inflammatory cytokines, such as IL-1β, IL-12p70, TNF-α, IL-6, IL-10 and IL-17, in chronic idiopathic urticaria[5,6].

Crohn's disease is also a disease with autoimmune involvement, and there is evidence for an altered cytokine milieu leading to mucosal inflammation. Although the exact mechanism of Crohn's disease has not been determined, recent studies have shown that T-cell production of certain cytokines play a strong role in the pathophysiology of Crohn's disease[7-11].

A thorough literature review has revealed very few



Figure 1 Angioedema and urticaria of the face and abdomen. A: Face; B: Abdomen.

case reports of urticaria or angioedema associated with IBDs. These include cases of Hereditary angioedema associated with Crohn's disease[12,13], angioedema of the small intestine masquerading as Crohn's disease[14,15], and a single case of chronic urticaria without angioedema in a patient who was subsequently diagnosed with Crohn's disease[16]. There has also been a case report of chronic urticaria and ulcerative colitis[17].

One possible common thread in the pathophysiology of chronic idiopathic urticaria and Crohn's disease is the derangement in cytokine levels, in particular, IL-17 and TNF-α. The IL-17 cytokines are T-cell derived cytokines that stimulate various cells to secrete cytokines and chemokines, and therefore play an important role in many autoimmune diseases[7], The Th17 CD4+ T cells produce a distinct set of cytokines (IL-17A, IL-17F, IL-6, IL-22 and IL-26) which enhance immune and host defenses. IL-17A plays a role in the expansion and recruitment of innate immune cells (neutrophils), and interacts with toll-like receptor ligands, IL-1 β, and TNF-α to enhance inflammatory reactions. IL-17F induces the secretion of other inflammatory cytokines such as IL6, IL-8 and LIF. It has been shown that Il-17A positive cells are increased in the inflamed mucosa of IBD patients[9], and IL-17F mRNA expression is elevated in the mucosa of Crohn's disease patients[8].

Infliximab and adalimumab are anti-TNF-α agents that block the inflammatory cascade. Both of these agents have been found to be effective in the treatment of Crohn's disease[18,19]. Given the similarity in cytokine derangements found in chronic idiopathic urticaria and in Crohn's disease, anti-inflammatory medications that target these cytokines should be effective in both conditions. Anti-TNF-α agents are still experimental for the treatment of urticaria, and have been tried in patients with various forms of urticaria, with a few case reports that have indicated successful treatment[20].

In summary, this is the first known case report of chronic idiopathic urticaria with angioedema coexistent with Crohn's disease that was successfully treated with anti-TNF-α agent. We hypothesize that the derangement in cytokines, especially IL-17 and TNF-α, may be the reason the anti-TNF-α agents were successful, and that there may be a common pathophysiology between autoimmune diseases. Patients with IBD and concurrent angioedema or urticaria could have their cytokine levels checked and compared to see if there is any trend. These levels could be checked before and after treatment with biologics to confirm the biologic effect on the cytokine milieu in these two diseases. This case brings to attention the need for further research looking into the changes in the cytokine milieu as potential targets for treatment.

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CASE REPORT

Catheter-directed thrombolysis in inflammatory bowel diseases: Report of a case

Péter Ilonczai, Judit Tóth, László Tóth, István Altorjay, Zoltán Boda, Károly Palatka

Péter Ilonczai, Zoltán Boda, Division of Haemostasis, 2nd Department of Medicine, Medical and Health Science Centre, University of Debrecen, 4032 Debrecen, Hungary
Judit Tóth, Euromedic Diagnostics Hungary Ltd., Medical and Health Science Centre, University of Debrecen, 4032 Debrecen, Hungary
László Tóth, Department of Pathology, Medical and Health Science Centre, University of Debrecen, 4032 Debrecen, Hungary
István Altorjay, Károly Palatka, Division of Gastroenterology, 2nd Department of Medicine, Medical and Health Science Centre, University of Debrecen, 4032 Debrecen, Hungary
Author contributions: Ilonczai P collected the data and wrote the article, participated in the treatment of the patient in the intensive care unit; Tóth J carried out the endovascular intervention and made the angiographic pictures; Tóth L analysed the histology; Boda Z supported and supervised the thrombolytic treatment; Altorjay I and Palatka K observed the patient, took care and managed of his IBD and performed the endoscopic investigations, and coordinated the treatment and revised critically the article.
Correspondence to: Károly Palatka, MD, PhD, Division of Gastroenterology, 2nd Department of Medicine, Medical and Health Science Centre, University of Debrecen, 4032 Debrecen, Hungary. palatka@med.unideb.hu
Telephone: +36-52-255152 Fax: +36-52-255152
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Abstract

In patients with inflammatory bowel diseases (IBD) the prevalence of thrombosis is 6.2%, the average incidence of thromboembolism (TE) is 3.6 times higher compared to normal population. The TE is a common extraintestinal complication of IBD, squarely associated with the IBD activity. The application of anticoagulant and thrombolytic therapy in severe IBD is an unresolved issue. Herein we report the first case in literature of an active IBD patient with an upper limb acute arterial occlusion and successful catheter-directed thrombolysis (CDT). A 46-year-old male patient is reported who had Crohn's disease for 10 years. His right hand suddenly became cold and painful. Angiography proved acute occlusion of the brachial and radial artery. Vascular surgery intervention was not applicable. Endoscopy showed extended, severe inflammation of the colon. Despite the severe endoscopic findings, frequent bloody stools and moderate anaemia, CDT with recombinant tissue plasminogen activator was performed. The control angiography proved improvement, the radial artery pulse appeared. No bleeding complication was observed. This case supports that CDT-after careful estimation of the bleeding risk-can be effective and safe in patients with severe or life-threatening TE and active IBD.

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Key words: Inflammatory bowel disease; Crohn's disease; Thromboembolism; Catheter-directed thrombolysis; Gastrointestinal haemorrhage

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) the two main forms of inflammatory bowel diseases (IBD) are systemic disorders often associated with extraintestinal

manifestations, complications, and other immune mediated disorders. These chronic inflammatory conditions are characterized by a hypercoagulable state and prothrombotic condition, and accompanied by abnormalities in coagulation. Thromboembolism (TE) is a significant cause of morbidity and mortality in patients with IBD. Recent data suggest that TE is a disease-specific extraintestinal manifestation of IBD, which is developed as the result of multiple interactions between acquired and genetic risk factors. The incidence of systemic thromboembolic events in IBD patients ranges from 1%-7.7%[1], patients having a 3-4-fold overall increase risk for venous TE compared to control population[2].

The application of anticoagulant and thrombolytic therapy in severe IBD is an unresolved issue. Generally the management of TE in IBD patients not differs from treatment in non-IBD patients, although there are currently no clear guidelines for the management of IBD-related TE[3].

CASE REPORT

A 46-year-old male with a past medical history of 10 years' CD, was presented with symptoms of relapse of IBD: weight loss, lower abdominal pain, bloody diarrhoea and arthralgia (CD activity index: 345). He was treated with high dose prednisone due to severe activity of IBD one year before this event. Actually he was on a treatment with azathioprine (2.5 mg/bwkg) and mesalazine (3 g/d), on a stable dose during the last 1 year. He also complained about an acute onset of pain and coldness of his right hand. The physical examination revealed to an acute arterial occlusion of the right hand. No provoking factor could have been detected. The colour-Doppler imaging and the angiography proved the acute occlusion of the right brachial and radial artery (Figure 1A and B). Vascular surgery intervention was not applicable. The laboratory findings showed anaemia (haemoglobin was 85 g/L) and thrombocytosis (platelet count was 409 g/L normal 150-400 g/L), the D-dimer test was positive (1.0 normal < 0.5 Feu/μL). Liver and kidney panels were normal. An acutely performed endoscopy showed extended, severe inflammation of the colon with deep ulcers and signs of chronic activity involving the sigmoid colon. Considering the severity of the occlusion and the risk of a potential limb-loss catheter-directed thrombolysis (CDT) was suggested, despite the severe endoscopic findings, frequent bloody stools and moderate anaemia. After 24 h from onset of symptoms of arterial occlusion endovascular arterial intervention was performed: 5 mg bolus of radial artery pulse appeared (rt-PA) (Actilyse injection, Boehringer Ingelheim International) was injected into the thrombus, followed by a continuous rt-PA infusion through the catheter in a dose of 0.1 mg/bwkg per hour. The treatment was carried on for 9 h and proceeded with enoxaparin (60 mg QD, subcutaneously). The pain and coldness quickly decreased, the radial pulse became palpable. The control angiography proved the improvement (Figure 1C and D). Despite the high risk of bleed no bleeding complication was observed. Echocardiography found no source of an embolus. Low-molecular-weight heparin (LMWH) prophylaxis was applied for 3 mo (enoxaparin, 60 mg OD, subcutaneously). No congenital thrombophilia was proved (antithrombin-Ⅲ: 120% normal: 80-120; protein C: 132% normal: 70-130; protein S: 93% normal: 60-140; activated protein C resistance: 2.51 normal: > 2; FVLeiden: wild type; Factor Ⅱ 20210A: wild type; Factor Ⅷ: 188% normal: 60-150). Neither lupus anticoagulant nor antiphospholipid autoantibody was detectable. The level of homocystein was also normal. The relapse of the CD was treated with anti-tumor necrosis factor-α (anti-TNF-α) antibody (infliximab). One year after the biological treatment and continuous LMWH prophylaxis the patient is in remission of IBD and has a complete recovery of the right upper limb arterial circulation.

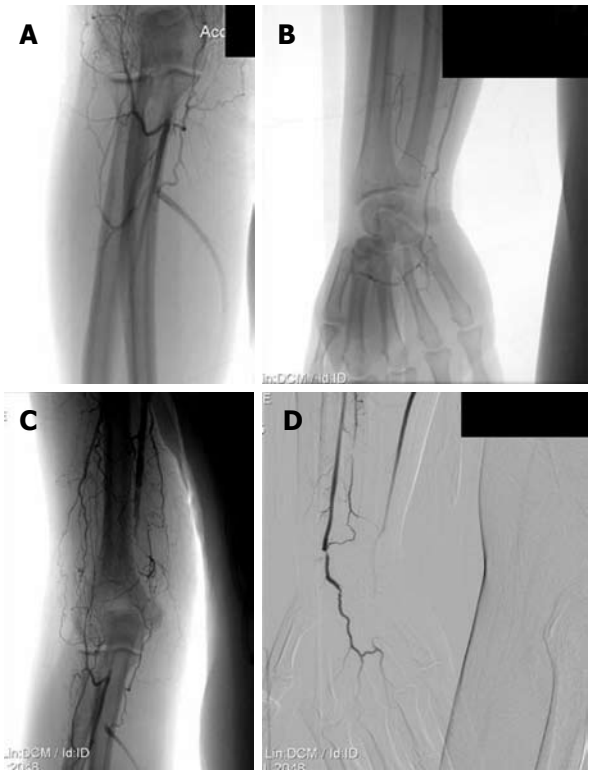


Figure 1 Angiographic pictures. A: Occlusion of the right brachial; B: Occlusion of the radial artery; C and D: Angiographic control after local thrombolytic treatment.

DISCUSSION

We report a case of a peripheral arterial TE of the right upper limb in a 46-year-old patient with acute relapse of CD. In the background no provoking factors could have been detected. Thrombophilia evaluation was normal and a source of an embolus was undetectable either. The background of the hypercoagulability that is present in IBD is the altered platelet/endothel cell function and interaction, the elevated level of coagulation factors and the decreased activity of fibrinolysis. In patients with IBD the prevalence of thrombosis is 6.2%, the average

incidence of TE is 3.6 times higher compared to normal population[1]. TE events occur mainly in the venous circulation[1,4], but can also develop in the arterial circulation[5]. Deep vein thrombosis and pulmonary embolism are the most common types of TE, but thromboses are also reported in unusual sites such as cerebral, innominate, retinal, hepatic and mesenteric veins[2,6]. Arterial thromboembolic complications occur less frequently[7] and the majority of cases seem to occur post surgery[8]. Patients with IBD have a markedly increased risk of acute mesenteric ischemia. IBD patients are more likely to have cardiac arterial thromboembolic disease, regardless of diagnosis or sex[9]. CD has an increased risk for cerebral arterial thromboembolic disease with some differences regarding the sex[10]. While the risk of gastrointestinal bleeding has to be considered in each case, most patients tolerate anticoagulants at full dose. Thrombolytic therapy is generally preserved for massive, life-threatening thrombosis. So far, only 17 cases of local thrombolytic treatment in IBD patient have been reported[11,12]. Until now there has been no case reported about upper limb arterial occlusion and CDT in IBD. In conclusion, moderate-severe active CD has to be considered as a risk factor for systemic TE[13]. Practice guidelines strongly recommend the use of thrombosis-prophylaxis in active IBD[3]. Arterial thrombosis is a rare, but severe complication of IBD. Considering the severity of the occlusion, in cases of life-threatening TE CDT could be an effective and safe intervention.

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CASE REPORT

Development of perianal ulcer as a result of acute fulminant amoebic colitis

Takayuki Torigoe, Yoshifumi Nakayama, Koji Yamaguchi

Takayuki Torigoe, Yoshifumi Nakayama, Koji Yamaguchi, Department of Surgery 1, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan

Author contributions: Torigoe T and Nakayama Y performed surgery and clinical follow-up; Torigoe T wrote the manuscript; and Yamaguchi K revised the manuscript.

Correspondence to: Takayuki Torigoe, MD, PhD, Department of Surgery 1, School of Medicine, University of Occupational Environmental Health, 1-1 Iseigaoka, Yahata-nishi-ku, Kitakyushu 807-8555, Japan. cyotori@yahoo.co.jp
Telephone: +81-93-6031611 Fax: +81-93-6032361
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Abstract

We report a case of acute fulminant amoebic colitis that resulted in the development of a perianal ulcer in a 29-year-old Japanese homosexual man with acquired immunodeficiency syndrome (AIDS). The patient was admitted to our hospital with a persistent perianal abscess that was refractory to antibiotic therapy administered at another hospital. On admission, we observed a giant ulcer in the perianal region. At first, cytomegalovirus colitis was suspected by blood investigations. Ganciclovir therapy was initiated; however, the patient developed necrosis of the skin around the anus during therapy. We only performed end-sigmoidostomy and necrotomy to avoid excessive surgical invasion. Histopathological examination of the surgical specimen revealed the presence of trophozoite amoebae, indicating a final diagnosis of acute fulminant amoebic colitis. The patient's postoperative course was favorable, and proctectomy of the residual rectum was performed 11 mo later. Amoebic colitis is one of the most severe complications affecting patients with AIDS. Particularly, acute fulminant amoebic colitis may result in a poor prognosis; therefore, staged surgical therapy as a less invasive procedure should be considered as one of the treatment options for these patients.

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Key words: Acute fulminant amoebic colitis; Perianal ulcer; Acquired immunodeficiency syndrome; Cytomegalovirus colitis; Staged surgery

Peer reviewer: Dr. Xiaoyun Liao, Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Room JF-208E, Boston, MA 02215, United States

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INTRODUCTION

Amoebiasis is a parasitic infection caused by the protozoan *Entamoeba histolytica*, which principally affects the colon and liver. A majority of infected patients remain asymptomatic; however, the development of acute fulminant amoebic colitis is often fatal, with a very high mortality rate of 70%-100%[1,2] because of serious complications like colonic perforation[3,4] and necrotizing colitis[5]. On the other hand, the formation of a perianal ulcer due to acute fulminant amoebic colitis is very rare[6]. We describe the case of a 29-year-old homosexual man with acquired immunodeficiency syndrome (AIDS), who developed a perianal ulcer as a result of acute fulminant amoebic colitis.

CASE REPORT

A 29-year-old Japanese homosexual man was admitted to our hospital with a complaint of spiking pyrexia and anal pain with a perianal abscess, which was refractory to an-



Figure 1 A giant ulcer covered with necrotic tissue is identified in the perianal region.

tibiotics therapy administered at another hospital. On admission, we observed a giant ulcer in the perianal region (Figure 1). A diagnosis of AIDS was made on the basis of serum human immunodeficiency virus (HIV) antigen positivity and an increased HIV viral load (90 000 RNA copies/mL); his condition was further complicated by *pneumocystis carinii* infection. Moreover, cytomegalovirus (CMV) colitis was suspected from the occurrence of severe bloody diarrhea and the presence of serum antigenemia. Ganciclovir therapy was initiated without performing a colonoscopy because of patient refusal. However, he developed infected necrosis of the skin around the anus and over the perianal ulcer during therapy. Laboratory investigations revealed the following: white blood cell count, 7500/mm^3; hemoglobin, 9.7 g/dL; platelets, 545 000/mm^3; and serum C-reactive protein, 13.1 mg/dL. Abdominal computed tomography (CT) revealed an abscess formed due to necrosis and extending from the perianal region to the lower rectum (Figure 2). We therefore ruled out chances of anus preservation and performed only end-sigmoidostomy and necrotomy of the skin tissue from around the anus to the lower rectum. This was done to avoid excessive surgical invasion because of a significant decrease in the CD4-positive lymphocyte count (52 cells/mm^3). Macroscopic findings of the resected colon revealed multiple deep ulcerations covered with yellow necrotic plaques (Figure 3). Histopathological examination of the surgical specimen revealed trophozoite amoebae that had ingested red blood cells in the resected colon and necrotic tissue, leading to in a final diagnosis of acute fulminant amoebic colitis (Figure 4). Furthermore, the serological test for *Entamoeba histolytica* was also positive. Typical cytomegalic intranuclear inclusion bodies were not observed in the endothelial cells of the resected specimen. Soon after receiving the pathological diagnosis of amoebiasis, metronidazole therapy was initiated. The patient's postoperative course was uneventful because of intensive care. Antiretroviral therapy was also prescribed. The HIV viral load gradually decreased to below the detection limit of the HIV-RNA assay, and the CD4-positive lymphocyte count increased to 112 cells/mm^3. On the basis of these findings, proctectomy of the residual rectum was performed 11 mo later.

DISCUSSION

Amoebiasis, which is caused by the protozoan *Entamoeba histolytica* (*E. histolytica*), is the second most frequent parasitosis worldwide, particularly in tropical and subtropical areas[7]. Recently, the risk of amoebiasis has also increased in developed countries, particularly among travelers returning from endemic areas and homosexual men, including those with HIV infection[8,9]. Interestingly, 13.4%-20.4% Japanese homosexual men test positive for amoebic antibodies[10]. However, acute fulminant amoebic colitis is uncommon, and Adams *et al*[11] reported that acute fulminant colitis developed in only 3% of 3013 patients with amoebic colitis in South Africa. The outcome of this condition is very poor, with a high mortality rate of 70%-100%[1,2] because of serious complications like colonic perforation[3,4], necrotizing colitis[5], and toxic megacolon[12]. Perianal ulcers caused by acute fulminant amoebic colitis, such as that observed in the present case, are very rare and occur in only 0.04% patients[11]. Especially in homosexual men, the pathogen can easily invade the anal epithelium, thus resulting in the formation of a perianal ulcer.

Amoebic colitis can be diagnosed by the identification of *E. histolytica* in the stool or in colonic tissue removed by colonoscopy. Testing for serum antibodies against *E. histolytica* is also useful, with a high sensitivity of 85%[13]. In the present case, we could not perform colonoscopic examination because the patient did not consent to this procedure. He was initially treated by ganciclovir therapy after CMV colitis was suspected because of the occurrence of severe bloody diarrhea and the presence of serum antigenemia; however, he responded poorly to this treatment. Because the final diagnosis of amoebic colitis was only made after surgery, metronidazole therapy, which is the standard treatment for amoebic colitis, was delayed. It is very difficult to distinguish these two diseases by nonspecific clinical manifestations, such as diarrhea, abdominal pain, and gastrointestinal bleeding; moreover, a few cases of mixed infection with *E. histolytica* and CMV have been previously reported[14-16]. However, it should be considered that amoebic colitis is one of the causes of perianal ulcers in homosexual men. The primary treatment of amoebic colitis is metronidazole; nevertheless, surgical treatment is necessary in cases of perforative peritonitis[1,2], necrotizing colitis[5], and toxic megacolon[12] despite high mortality and poor prognosis. In the present case, the perianal ulcer may have developed as a result of acute fulminant amoebic proctitis, which may be attributed to homosexuality. Several reports have advocated less invasive surgical procedures, such as diverting colostomy or ileostomy and simple closure of the perforation, because of the high mortality rate following bowel resection surgery[17,18]. On the other hand, resection to resolve the septic focus and stoma creation should be considered one of the treatment options, depending on the general status of the patient[19]. We performed only end-sigmoidostomy and necrotomy of the skin tissue from around the anus

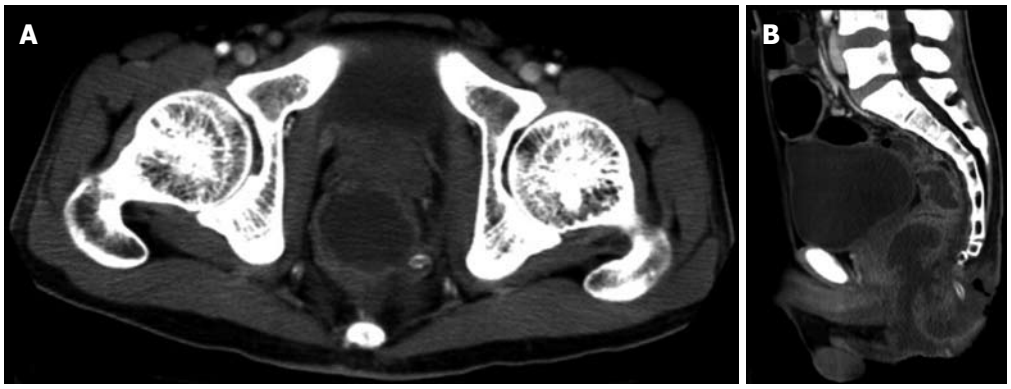


Figure 2 Abdominal computed tomography revealing an abscess formation of the perianal region. A: The axial view shows an abscess of the perianal region due to necrosis; B: The sagittal view shows an abscess formation extending from the perianal region to the lower rectum.



Figure 3 Macroscopic findings of the resected colon following end-sigmoidostomy show numerous deep ulcerations covered with yellow necrotic plaques.

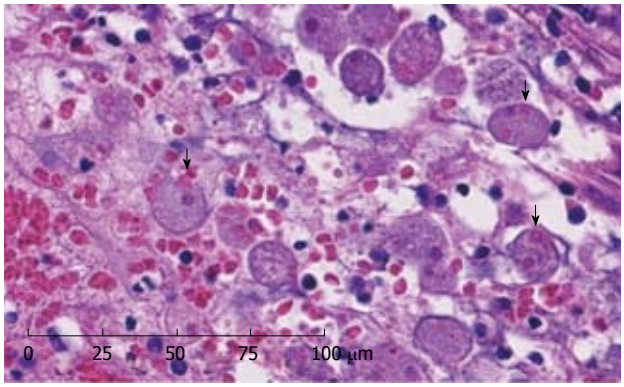


Figure 4 Histopathological findings of the surgical specimen show trophozoite amoebae that have ingested red blood cells (arrows) (HE staining).

to the lower rectum to avoid excessive surgical invasion because of a significant decrease in the CD4-positive lymphocyte count due to AIDS. Proctectomy of the residual rectum was performed after the recovery of his immunocompetence 11 mo later. The patient's postoperative course following staged surgical treatment was favorable, and he has survived for 2 years since the first surgery.

Because of the rarity of this disease, the effectiveness of invasive surgical procedures for the treatment of acute fulminant amoebic colitis needs further investigation.

In summary, we described an extremely rare case of acute fulminant amoebic colitis that resulted in the development of a perianal ulcer in a homosexual man with AIDS. He was successfully treated by staged surgical treatment. Amoebiasis is the second most frequent parasitosis worldwide, but almost all of these infections are asymptomatic. However, acute fulminant amoebic colitis has a poor prognosis in immunocompromised patients, particularly those with AIDS. Early diagnosis, opportune surgical procedures, and intensive care for sepsis appear to decrease mortality. Therefore, amoebic colitis must be kept in mind when perianal ulcers manifest in immunocompromised patients; moreover, minimally invasive, staged surgical treatment should be considered in these patients.

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CASE REPORT

A pedunculated polyp-shaped small-bowel lymphangioma causing gastrointestinal bleeding and treated by double-balloon enteroscopy

Akihiko Kida, Koichiro Matsuda, Satoshi Hirai, Akiyoshi Shimatani, Yousuke Horita, Katsushi Hiramatsu, Mitsuru Matsuda, Hidero Ogino, Shin Ishizawa, Yatsugi Noda

Akihiko Kida, Koichiro Matsuda, Satoshi Hirai, Akiyoshi Shimatani, Yousuke Horita, Katsushi Hiramatsu, Mitsuru Matsuda, Hidero Ogino, Yatsugi Noda, Department of Internal Medicine, Toyama Prefectural Central Hospital, Toyama 930-8550, Japan
Shin Ishizawa, Department of Clinical Pathology, Toyama Prefectural Central Hospital, Toyama 930-8550, Japan
Author contributions: Kida A, Matsuda K, Hiramatsu K, Ishizawa S and Noda Y contributed equally to this work; Kida A, Matsuda K, Hirai S, Shimatani A, Horita Y, Hiramatsu K, Matsuda M, Ogino H, Ishizawa S and Noda Y designed research; Kida A, Matsuda K, Hiramatsu K, Ishizawa S and Noda Y performed the research; Kida A and Noda Y contributed to new reagents/analytic tools; Kida A analyzed the data; and Kida A and Noda Y wrote the paper.
Correspondence to: Akihiko Kida, MD, Department of Internal Medicine, Toyama Prefectural Central Hospital, 2-2-78 Nishinagae, Toyama-city, Toyama 930-8550, Japan. kidaakihiko@yahoo.co.jp
Telephone: +81-764-241531 Fax: +81-764-220667
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Abstract

We report a rare case of a small-bowel lymphangioma causing massive gastrointestinal (GI) bleeding that we successfully diagnosed and treated using double-balloon enteroscopy (DBE). An 81-year-old woman suffering from repeated GI bleeding of unknown origin underwent a capsule endoscopy at a previous hospital. She was suspected of having bleeding from the jejunum, and was referred to our department for diagnosis and treatment. An oral DBE revealed a 20 mm × 10 mm, regularly surfaced, white to yellowish, elongated, pedunculated jejunal polyp with small erosions at 10 cm distal to the ligament of Treiz. Since no other source of bleeding was identified by endoscopy in the deep jejunum, an endoscopic polypectomy (EP) was performed for this lesion. A subsequent histopathological examination of the resected polyp showed clusters of lymphatic vessels with marked cystic dilatation in the submucosa and the deep layer of the lamina propria mucosae. These characteristics are consistent with the typical features of small-bowel lymphangioma with erosions. Although clipping hemostasis was performed during EP, re-bleeding occurred. Finally, a complete hemostasis was achieved by performing an additional argon plasma coagulation.

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Key words: Small-bowel lymphangioma; Gastrointestinal bleeding; Double-balloon enteroscopy

Peer reviewers: Dr. Weekitt Kittisupamongkol, Hua Chiew Hospital, 665 Bumrungmuang Road, Bangkok 10100, Thailand; Marcela Kopacova, MD, PhD, Professor, 2nd Department of Internal Medicine, Charles University in Praha, Faculty of Medicine at Hradec Král, University Teaching Hospital, Sokolska 581, 50005 Hradec Kralove, Czech; Satoshi Osawa, Assistant Professor, The First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

Kida A, Matsuda K, Hirai S, Shimatani A, Horita Y, Hiramatsu K, Matsuda M, Ogino H, Ishizawa S, Noda Y. A pedunculated polyp-shaped small-bowel lymphangioma causing gastrointestinal bleeding and treated by double-balloon enteroscopy. *World J Gastroenterol* 2012; 18(34): 4798-4800 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i34/4798.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i34.4798

INTRODUCTION

A small-bowel lymphangiomas is a rare benign tumor of the lymphatic system but has been previously reported as

a cause of significant gastrointestinal (GI) bleeding[1]. Recently, double-balloon enteroscopy (DBE) has been used for the diagnosis and treatment of obscure GI bleeding. We here report a rare case of a pedunculated polyp-shaped small-bowel lymphangioma causing massive GI bleeding that we successfully treated using DBE. In this patient, DBE therapy was performed using a combination of endoscopic polypectomy (EP), hemoclipping, and argon plasma coagulation (APC). This is a particularly unusual and interesting case as it has not only revealed a further rare form of small-bowel lymphangioma but also that DBE is an effective procedure for not only the diagnosis but also the treatment of this disorder. We conclude from our findings in this case that future differential diagnoses of GI bleeding of unknown origin should include small-bowel lymphangioma even though it is a rare condition.

CASE REPORT

An 81-year-old woman was treated for chronic heart failure and myeloid dysplasia (MDS). Since MDS was the underlying disease in this patient, her Hb levels were low at approximately 9.0 g/dL and in February 2011 decreased to 5.0 g/dL. Although upper GI endoscopy, lower GI endoscopy, and computed tomography procedures were performed, no source of bleeding could be identified. Subsequently, she had frequent episodes of melena and received a total of 20 transfusions of packed red cells (2800 mL), and underwent capsule endoscopy using a PillCam SB (Given Imaging, Ltd., Israel). This testing raised suspicions that the source of her bleeding was from the jejunum. She was subsequently referred to our hospital for further diagnosis and treatment.

Since the lesion in this patient was located in the jejunum, antegrade DBE (Fujinon EN-450T5/W, Fujinon Corp., Saitama, Japan) was performed under intravenous propofol anesthesia. As a result, a collection of blood was observed at 10 cm distal to the ligament of Treitz, and a 20 mm × 10 mm white to yellowish, elongated, pedunculated jejunal polyp with erosions was identified (Figure 1). The DBE was advanced as far as possible to examine the distal jejunum and ileum, but no other source of bleeding was evident. After the placement of two hemoclips at the polyp base, EP was performed (Figure 2). The total time taken to perform the examinations and treatment, including the examination of the distal jejunum and the EP procedure, was 90 min. Surgery was completed without complications. Subsequent histopathological examination of the resected polyp revealed clusters of lymphatic vessels with marked cystic dilatation in the submucosa and in the deep layer of the lamina propria mucosae (Figure 3). These findings were consistent with the typical features of small-bowel lymphangioma with erosions. Following treatment, re-bleeding occurred twice at one and two months post-surgery. An antegrade DBE revealed that this occurred from the polypectomy site on both occasions. Hemoclipping and APC, after a submucosal saline injection to prevent perforation, were performed to achieve hemostasis at each instance of bleeding (Figure 4). No subsequent progression of anemia or recurrence of melena was observed in the patient.

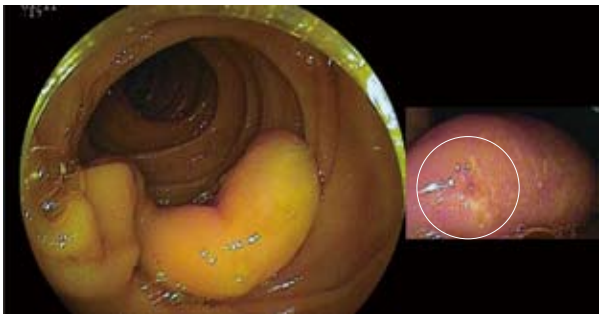


Figure 1 A 20 mm × 10 mm, white to yellowish, elongated, pedunculated jejunal polyp with erosions was detected in the patient following a double balloon enteroscopy.

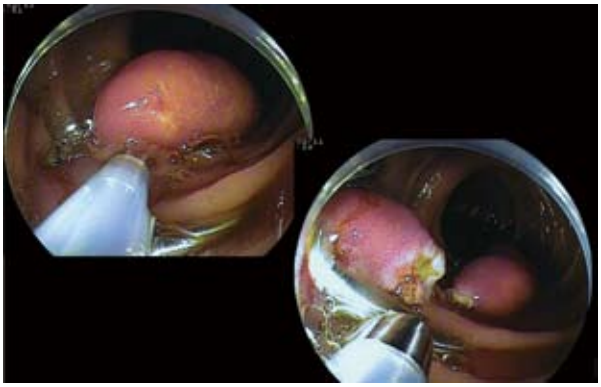


Figure 2 An endoscopic polypectomy was performed after the placement of two hemoclips at the polyp base.

DISCUSSION

Small-bowel lymphangiomas are commonly asymptomatic, but sometimes present with symptoms such as GI bleeding, abdominal pain, intussusception, and protein-losing gastroenteropathy[1,2]. Only 15 cases, including our current patient, of small-bowel lymphangioma with GI bleeding have been reported since 1967[1,3-9]. Of these, five individuals including our present case were diagnosed using DBE from 2007 onward[4-6]. The form of small-bowel lymphangioma revealed in the four previous cases was either a circumferential polyp with adjacent intestinal wall thickening or a flat or sessile polyp. The pedunculated polyp identified in our present case is therefore an extremely rare finding for a small-bowel lymphangioma.

Two reported patients, including the current case, have been treated endoscopically using DBE. Li *et al*[5] reported the treatment of a flat, hemorrhagic lesion by APC after a submucosal epinephrine injection to stop an occurrence of bleeding, thereby achieving complete hemostasis. However, as our present patient had a pedunculated polypoid lesion, EP was first performed by additional hemoclipping. However, re-bleeding occurred in our case, and hemostasis was finally achieved by APC. In

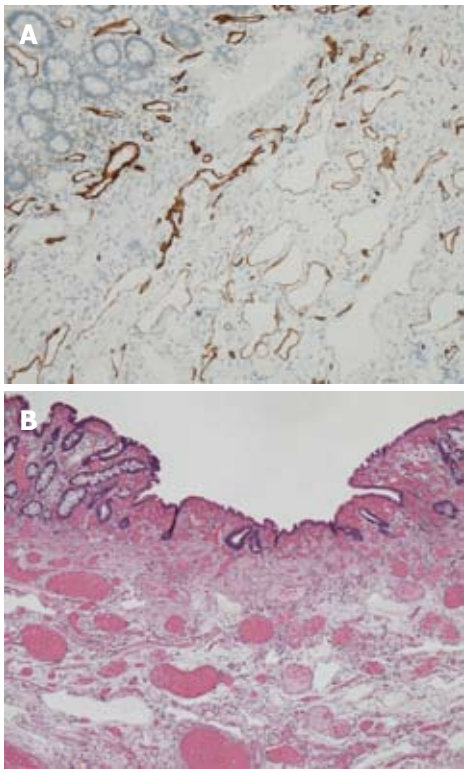


Figure 3 Histological view of the tumor revealing clusters of lymphatic vessels with a marked cystic dilatation in the submucosa and deep layer of the lamina propria mucosae. A: Staining was performed with D2-40; B: Staining was performed with hematoxylin and eosin. These characteristics are consistent with the typical features of small-bowel lymphangioma with erosions.

terms of the cause of this re-bleeding, we speculate that a residual lymphangioma was present at the polypectomy site. Another previous case report has described a giant pedunculated angiolipofibroma of the distal duodenum for which EP was performed using an endoloop[10]. In this case, re-bleeding did not occur, but it was not certain from this that complete *en bloc* resection could be achieved by EP using an endoloop in cases of small-bowel lymphangioma.

We conclude from our present analysis that in cases of small-bowel lymphangioma causing GI bleeding, DBE therapy with a combination of EP, hemoclipping, and APC, would likely be beneficial approaches to treatment.

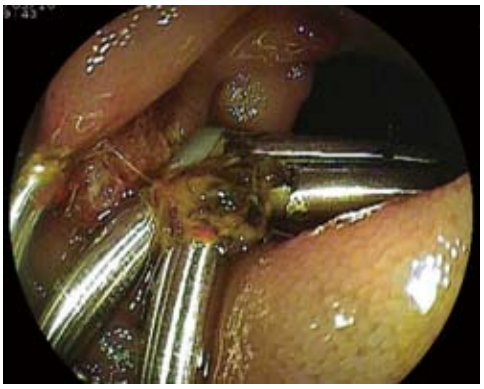


Figure 4 Lesion after treatment with hemoclipping and argon plasma coagulation.

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Imran Hassan, MD, Assistant Professor, Department of Surgery, SIU School of Medicine, 701 North Rutledge, PO Box 19638, Springfield, IL 62794, United States

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Kenji Miki MD, Department of Surgery, Showa General Hospital, 2-450 Tenjin-cho, Kodaira, Tokyo 187-8510, Japan

Yasushi Matsuzaki, Associated Professor, Division of Gastroenterology and Hepatology, Graduate School of Comprehensive Human Sciences and University Hospital, 1-1-1, Tennodai, Tsukuba 305-8575, Japan

Dr. Lucia Ricci Vitiani, Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy

Liang-Shun Wang, MD, Professor, Vice-Superintendent, Shuang-Ho Hospital, Taipei Medical University, No. 291, Jhongjheng Rd., Jhonghe City, New Taipei City 237, Taiwan, China

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

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Pancreas Club Meeting
San Diego, CA 92101, United States

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PancreasFest 2012
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September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
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September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

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September 8-9, 2012
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Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

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The Liver Meeting
San Francisco, CA 94101, United States

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Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
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Organization as author

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
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EDITORIAL OFFICE
Jian-Xia Cheng, Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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EDITORIAL

Biliary cysts: Etiology, diagnosis and management

Beata Jabłońska

Beata Jabłońska, Department of Digestive Tract Surgery, University Hospital of the Medical University of Silesia, 40-752 Katowice, Poland
Author contributions: Jabłońska B wrote this paper.
Correspondence to: Beata Jabłońska, MD, PhD, Department of Digestive Tract Surgery, University Hospital of the Medical University of Silesia, Medyków 14 St, 40-752 Katowice, Poland. bjablonska@poczta.onet.pl
Telephone: +48-32-7894251 Fax: +48-32-7894251
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Abstract

Biliary cysts (BC) are rare dilatations of different parts of a biliary tract. They account for approximately 1% of all benign biliary diseases. BC occur the most frequently in Asian and female populations. They are an important problem for pediatricians, gastroenterologists, radiologists and surgeons. Clinical presentation and management depend on the BC type. Cholangiocarcinoma is the most serious and dangerous BC complication. The other complications associated with BC involve cholelithiasis and hepatolithiasis, cholangitis, acute and chronic pancreatitis, portal hypertension, liver fibrosis and secondary liver cirrhosis and spontaneous cyst perforation. Different BC classifications have been described in the literature. Todani classification dividing BC into five types is the most useful in clinical practice. The early diagnosis and proper treatment are very important, because BC are associated with a risk of carcinogenesis. A malignancy risk increases with the age. Radiological investigations (ultrasonography, computed tomography, endoscopic retrograde cholangiopancreatography and magnetic resonance cholangiopancreatography) play an important role in BC diagnostics. Currently, prenatal diagnosis using ultrasonography is possible. It allows to differentiate biliary disorders in fetals and to perform the early surgical treatment that improves results. In most patients, total cyst excision with Roux-Y hepaticojejunostomy is the treatment of choice. Surgical treatment of BC is associated with high success rate and low morbidity and mortality. The early treatment is associated with a lower number of complications. Patients following BC surgery require permanent and careful postoperative observation using laboratory and imaging investigations because of possibility of biliary anastomosis stricture and biliary cancer in tissue remnant.

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Key words: Biliary cysts; Biliary drainage; Hepaticojejunostomy

Peer reviewer: Tamir Miloh, MD, Associate Professor, Director of Pediatric Liver/Liver Transplantation Program, Division of Gastroenterology, Phoenix Children's Hospital, 1919 E Thomas Rd, Main Building, Second Floor, Phoenix, AZ 85016, United States

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INTRODUCTION

Biliary cysts (BC) are rare dilatations of different parts of a biliary tract. They account for approximately 1% of all benign biliary diseases. BC occur the most commonly in Asian populations and in females (with a female:male ratio of 3-4:1). Incidence of this pathology in Asian populations is 1:1000 and it is lower in western countries (1:100 000 to 1:150 000)[1-6]. They are usually a surgical problem of infancy or childhood; however in approximately 20% of cases they are recognized in adults[2]. The early diagnosis and proper treatment are very important, because BC are associated with a risk of carcinogenesis. According to the literature, biliary tract malignancy occurs in 2.5%-28% of patients with BC[3,4]. The risk of biliary cancer increases with the age. It is the lowest in the childhood in the first decade (< 1%)[6]. In the third decade the cancer

risk is > 10%[7,8]. According to the literature, cholangiocarcinoma is reported the most frequently at the age of 32 years in patients with BC (about 20 years earlier than in general population)[7]. There are reports of cholangiocarcinoma developing in adult many years after radical BC resection in infant[9]. Cholangiocarcinoma is the most serious and dangerous BC complication. The other complications associated with BC involve cholelithiasis and hepatolithiasis, cholangitis, acute and chronic pancreatitis, portal hypertension, liver fibrosis and secondary liver cirrhosis and spontaneous cyst perforation[8-12]. Nicholl *et al*[9] noted a higher number of complications in adults compared to children. Therefore, BC constitute an important problem for pediatricians, gastroenterologists, radiologists and surgeons. The role of pediatricians and gastroenterologists is a proper early diagnosis. Radiological investigations play an important role in BC diagnostics. Currently, the prenatal BC diagnosis using ultrasonography (USG) is possible[6,13,14].

Surgery is the treatment of BC. The goal of surgical treatment is to remove a cyst and to reconstruct the proper bile flow to the alimentary tract. In order to achieve this goal, different techniques are used. Roux-Y hepaticojejunostomy is the method of choice in most patients with BC[5-22].

ETIOLOGY AND PATHOGENESIS OF BC

Many theories explain the etiology and pathogenesis of BC[2]. In 1936, Yotsuyanagi supposed that BC arouse from inequality in the cellular proliferation of the biliary tract during the early fetal life[23]. Babbitts's theory of the "common channel" (1969) is the most widely accepted in the literature. According to this theory, the common cannel is formed by abnormal pancreaticobiliary junction (APBJ) of the pancreatic and bile ducts outside the ampulla of Vater. This condition leads to pancreaticobiliary reflux and mixing of the pancreatic and biliary juices and activations of pancreatic enzymes, because the maximal pressure within the pancreatic duct is two to three timed higher than within a biliary tract. Activated pancreatic enzymes cause inflammation and deterioration of the bile duct wall that leads to biliary dilatation[24]. A number of studies analyzed the levels of amylase within the bile duct. The higher amylase concentrations have been observed in patients with BC compared to the control groups. Also, association between the amylase level, earlier presentation and dysplasia grade in patients with BC has been reported in the literature[25,26]. Therefore, there is a theory, that the pancreaticobiliary reflux leads to inflammation and dysplasia in patients with BC[2,26-29]. APBJ occurs only in 50%-80% patients with BC. Another counterargument to this hypothesis is a fact, that immature neonatal acini do not produce sufficient pancreatic enzymes to explain antenatal BC[2,30,31]. The counterargument supporting Babbitt's theory is arbitral definition of the long common channel that depends on imaging modality and angles. Therefore, a defined length of a long common channel has been described from 10 mm to 45 mm in the literature. Therefore, APBJ can occur in more patients than radiological investigations show[2]. According to Okada *et al*[32], long common channel as any pancreaticobiliary junction is defined, if it lies outside of the duodenal wall and thus could result in pancreaticobiliary reflux and mixing. APBJ has been divided into three types by Komi *et al*[33]: (1) A right-angled union without an accessory pancreatic duct; (2) An acute-angled union without an accessory pancreatic duct; and (3) A right- or acute-angled union with an accessory pancreatic duct.

Table 1 Types of an anomalous pancreaticobiliary junctions (Komi classification)

Type name	Type description
Type Ⅰ	A narrowed common bile duct joins the pancreatic duct at a right angle
Ⅰ A	A slender common channel
Ⅰ B	A short or long ectatic common channel
Type Ⅱ	A pancreatic duct joins the common bile duct at an acute angle
Ⅱ A	A slender common channel
Ⅱ B	A short or long ectatic common channel
Type Ⅲ	It is complicated by a patent accessory pancreatic
Ⅲ A	A classic pancreas divisum
Ⅲ B	A pancreas divisum without a pancreatic duct
Ⅲ C	A patent accessory pancreatic duct with an intricate network
Ⅲ C1	A tiny communicating duct between the main and accessory ducts
Ⅲ C2	A communicating duct with the same caliber as the main and accessory ducts
Ⅲ C3	A total or partial dilatation of the ductal system

These types are subdivided into subtypes according to the shape of the common channel. The Komi's classification is presented in Table 1. The type of BC and clinical presentation can depend on the type of an angle of the anomalous ductal junction. A right-angled union is associated with cystic dilatation of the common bile duct. In patients with this type of union a palpable mass or jaundice as the main sign of disease are observed. An acute-angled union is associated with fusiform dilatation of the common bile duct. Patients with such dilatation usually complain on abdominal pain, and an erroneous diagnosis of acute pancreatitis is occasionally made. Type Ⅰ B, Ⅱ B, and Ⅲ junctions are associated with a dilated common channel and an accessory pancreatic duct. They are frequently complicated by relapsing pancreatitis leading to chronic pancreatitis[34]. Therefore, it's important to describe the type of APBJ. The type of APBJ is based on a cholangiopancreatographical picture[33-35]. The other theories regarding BC pathogenesis are less popular. According to Singham *et al*[2], biliary dilation can be a result of embryologic overproliferation of epithelial cells within solid bile ducts during the fetal life. According to Davenport and Basu, dilations are due to a lower number of neurons and ganglions in patients with BC that have been recognized pathologically. They suggested that round cysts were congenital with distal obstruction due to agan-

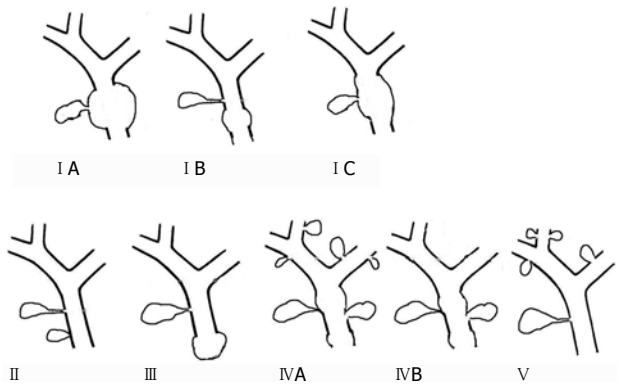


Figure 1 Types of biliary cysts according to Todani classification. Ⅰ: Solitary extrahepatic cyst (40%-85%): Ⅰ A: Common type; Ⅰ B: Segmental extrahepatic duct dilatation; Ⅰ C: Diffuse extrahepatic dilatation; Ⅱ: Extrahepatic diverticulum (2%-3%); Ⅲ: Choledochocele (1.4%-5.6%); ⅣA: Multiple extra- and intrahepatic cysts; ⅣB: Multiple extrahepatic cysts (18%-20%); Ⅴ: Multiple intrahepatic cysts (Caroli's disease) (rare).

glionosis and proximal dilation similarly to Hirschprung's disease. In this theory, chronic inflammation and clinical manifestation are caused by bile stasis within dilated bile ducts[2,31,36].

CLASSIFICATIONS OF BC

The first classification system for BC was proposed by Alonso-LEJ *et al*[37] in 1959. This classification involves three BC types: congenital cystic dilatation (the most common), congenital diverticulum of the common bile duct, and congenital choledochocele[37]. In 1977, Todani *et al*[38,39]modified BC classifications distinguishing five types (Figure 1). The most frequent (more than 90%) is type Ⅰ cyst. The type Ⅰ A cyst involves dilatation of the common bile duct, with marked dilatation of part or all of the extrahepatic bile ducts. The type Ⅰ B cyst involves segmental dilatation of the common bile duct, usually of its most distal part. The type Ⅰ C cyst involves fusiform dilatation of the common bile duct, along with diffuse, cylindric dilatation of the common hepatic duct and common bile duct. In 2011, Michaelides *et al*[40] reported and proposed a new BC variant and called it the Ⅰ D type. In this new variant, apart from the dilatation of the common hepatic and the common bile duct, dilatation of the central portion of the cystic duct was also observed, giving a bicornal configuration to the cyst. The type Ⅱ cyst is a diverticulum of the common bile duct usually arising laterally but may arise in the pancreatic portion. The type Ⅲ cyst (which is called a choledochocele) is a cystic dilatation of the intraduodenal portion of the common bile duct. According to some authors, choledochocele is different from other types of BC. In 2010, Ziegler *et al*[41], compared choledochocele with other types of BC (types Ⅰ, Ⅱ, Ⅳ and Ⅴ according to Todani classification). Based on study including 146 patients with BC (46 patients with choledochoceles), they concluded that classifications of choledochal cysts should not include choledochoceles. They reported that patients with choledochoceles differed from patients with other choledochal cysts with respect to age, gender, presentation, pancreatic ductal anatomy, and their management. Patients with cholechoceles were the most frequently male, older, complained with acute pancreatitis rather than jaundice or cholangitis, and were managed with endoscopic therapy. A pancreas divisum and a low risk of malignancy compared to other BC were observed with patients with choledochoceles. The type Ⅳ cyst involves dilatation of the intrahepatic and extrahepatic bile ducts. In the type ⅣA cyst, both the intrahepatic and extrahepatic bile ducts are dilated. The type ⅣB cyst involves dilatation of multiple segments of the extrahepatic bile ducts. The type Ⅴ cyst (Caroli's disease) involves dilatation of one or several segments of the intrahepatic bile ducts. First, it was described by Todd in 1818, but precise definition of this disorder was constructed by Jaques Caroli in 1958[41-45]. There are two descriptions (Caroli's disease and Caroli's syndrome) in the literature. Caroli's syndrome is the more serious condition compared to Caroli's disease. In Caroli's disease, cholangitis due to bile stasis within dilated intrahepatic bile ducts, is observed. In Caroli's syndrome, recurrent bouts of cholangitis due to bile stasis, hepatolithiasis, gallbladder stones lead to liver fibrosis with portal hypertension and liver failure. Caroli's syndrome can be associated with autosomal recessive polycystic kidney disease caused by a mutation of the *PKHD1* gene[46,47].

BC can be associated with the biliary atresia. Three biliary atresia (BA) types have been distinguished in the literature: 1 BA (atresia of the common bile duct), 2 BA (atresia of the common hepatic duct), and 3 BA (atresia of the porta hepatis)[18]. Muise *et al*[18], concluded that BA associated with BC formed a distinct subtype of BA, characterized by a preponderance of type 1 BA, a relatively good clinical outcome after surgery, and an absence of associated congenital anomalies. In 2004, Visser *et al*[48] proposed modified classification of BC and distinguished the following BC types: choledochal cyst, choledochal diverticulum, choledochocele, and Caroli's disease. All BC classifications are presented in Table 2.

CLINICAL PRESENTATION OF BC

The classic triad of clinical symptoms was described by Alonso-LEJ *et al*[37] and it involves right hypochondriac pain, palpable abdominal mass and jaundice. All clinical signs are observed only in 20%-30% patients[49]. Two of three clinical symptoms occur in 2/3 patients. The most frequently (in 80% of patients) clinical presentation appears before the age of 10 years[44]. Clinical presentation is different and it depends on the patient's age. According to the age, patients are divided into two groups: neonatal patients (under 12 mo) and adults (above 13 mo). Prenatally, BC are diagnosed incidentally during prenatal ultrasonography visualized as an intra-abdominal cystic mass[6,13,14]. In neonatal patients, obstructive jaundice

Table 2 Classifications of biliary cysts

Ref.	Classification
Alonso *et al*[37]	Congenital cystic dilatation
	Congenital diverticulum of the common bile duct
	Congenital choledochocele
Todani *et al*[38,39]	Ⅰ A Common type
	Ⅰ B Segmental extrahepatic duct dilatation
	Ⅰ C Diffuse extrahepatic duct dilatation
	Ⅱ Diverticulum type in the whole extrahepatic duct
	Ⅲ Choledochocele
	ⅣA Multiple cysts of the extra- and intra-hepatic ducts
	ⅣB Multiple cysts of the extrahepatic ducts
	Ⅴ Caroli's disease (intrahepatic bile duct cysts)
Vesser *et al*[48]	Choledochal cyst
	Choledochal diverticulum
	Caroli's disease

and abdominal mass are present the most frequently. Pain, fever and nausea are the most common for adult patients[44]. According to Lopez *et al*[21], pain is the commonest clinical sign in adults. Recurrent cholangitis and pancreatitis are complications of BC due to pancreaticobiliary reflux[49,50]. The other complications observed commonly in adults are the following: cholangiocarcinoma, cholecystolithiasis and cholecystitis, and liver cirrhosis[5]. Cholecystolithiasis and cholecystitis occur due to bile stasis in patients with BC. The secondary liver cirrhosis leads to different complications associated with portal hypertension such as upper gastrointestinal bleeding, splenomegaly and pancytopenia[49]. Portal hypertension can also occur without liver cirrhosis due to mechanical obstruction of the portal vein by the cyst[48]. An spontaneous BC rupture occurs in about 1%-12% of patients. It is presented as abdominal pain, peritonitis signs and sepsis[49,51]. Patients with choledochocele are usually asymptomatic. The Ⅲ BC type can be also presented by gastric outlet obstruction symptoms caused by direct obstruction of the duodenal lumen or intussusceptions[44]. It should be highlighted that BC are premalignant disorder. The overall reported risk of cancer is 10%-30% and it increases with age (from 0% in patients aged < 10 years to 75% in patients aged 70-80 years). Nicholl *et al*[9] reported direct correlation between the patient age and cancer risk: 0 year to 30 years (0%), 31 years to 50 years (19%), and 51 years to 70 years (50%). The histopathological types of cancer are the following: *adenocarcinoma* (73%-84%), *anaplastic carcinoma* (10%), *undifferentiated cancer* (5%-7%), *squamous cell carcinoma* (5%), and others (1.5%). The locations of cancer are the following: the extrahepatic bile duct (50%-62%), gallbladder (38%-46%), intrahepatic bile ducts (2.5%), the liver (0.7%), and the pancreas (0.7%)[49]. Todani *et al*[52] reported that 68% of cancers were associated with type-Ⅰ, 5% type-Ⅱ, 1.6% type-Ⅲ, 21% type-Ⅳ and 6% type-Ⅴ BC. Cholangiocarcinoma occurs as a result of chronic inflammation, cell regeneration and DNA breaks, leading to dysplasia[49,52]. The following molecular lesions have been reported in BC during carcinogenesis: microsatellite instability, k-ras mutations, expression of COX-2 and bcl-2, and increased telomerase activity that occur early and involvement of cyclin D1, beta-catenin, DPC-4/Smad4 and p53 that occur later[7].

DIAGNOSTICS OF BC

Laboratory investigations

Laboratory investigations may demonstrate mildly abnormal liver function and cholestasis tests (serum bilirubin, alkaline phosphatase, γ-glutamyltranspeptidase, alanine and aspartate aminotransferases) or amylase values, but these findings are not specific[34,49-56].

Radiological investigations

Imaging investigations are the most useful in BC diagnosis. The following radiological examinations are performed in order to visualize biliary dilatation: ultrasonography (USG) of the abdominal cavity, computed tomography (CT) of the abdominal cavity, and cholangiography[34,49-56].

USG of abdominal cavity

USG is the initial and easy to perform examination. It allows imaging of intrahepatic and extrahepatic bile ducts with measurement of the diameter of common bile duct or common hepatic duct and BC. It demonstrates (with the exception of type-Ⅲ and type-Ⅴ cysts) a cystic mass in the right upper quadrant (usually at the porta hepatis) separately from the gallbladder[49].

Use of USG in prenatal diagnosis

USG is very useful in prenatal diagnosis of BC. USG shows BC as a round intra-abdominal cystic mass located in the upper abdominal quadrant. Color Doppler USG shows no significant flow within the mass. Differential diagnosis of the cystic mass in prenatal USG should involve simple hepatic cysts, biliary atresia, ovarian, omental or mesenteric cysts, duodenal or gall bladder duplications, adrenal cysts, renal cysts, dilated loop of bowel, hydronephrotic renal pelvis and situs inversus. Proper early differential diagnosis between BC and another biliary disorder such as BA is very important, because BA needs an immediate surgery[6,13,14]. When the differential diagnosis of a cystic mass in the right upper quadrant is difficult to make by conventional USG alone, ultrasound-guided BC aspiration may serve as an alternative to the prenatal diagnosis of BC in the fetus[57,58]. Tanaka *et al*[14] compared imaging (USG and CT) and laboratory investigations in BC and BA in order to differentiate these two pathologies. They concluded that patients with biliary disorder smaller than 21 mm, direct bilirubin level higher than 2.5 mg/dL, and total bile acid level higher than 111 μmol/L in the neonatal period were more likely to have BA than BC and needed a surgery as soon as possible before irreversible liver cirrhosis. Okada *et al*[17] described the differential diagnosis between BC and BA by immunohistological examination using liver biopsy specimens.

They indicated that CD56-positive biliary duct cells were present in prenatally diagnosed type-1 cystic BA.

Cholangiography

Proper diagnosis requires demonstration of continuity of the cyst with the biliary tract, because it allows to differentiate BC from other intrabdominal cysts such as pancreatic pseudocysts, echinococcal cysts or biliary cystadenomas[49]. In order to show it, cholangiography is performed. There are the following methods of cholangiography: endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance-cholangiopancreatography (MRCP), percutaneous transhepatic cholangiography (PTC) and intraoperative cholangiography. These investigations allow to demonstrate the anatomy of biliary tree and define the BC type[49].

ERCP

ERCP is invasive investigation, but it has got therapeutic possibility. It accurately reveals the presence of any associated intraductal pathology or an APBJ. In the a type Ⅲ BC, it allows to perform a therapeutic papillotomy simultaneously[49].

MRCP

MRCP is a non-invasive procedure compared to ERCP. It is a favoured alternative to ERCP, but it has a lower accuracy in the detection of APBDJ and lacks the therapeutic possibility in case of the type Ⅲ BC. MRCP, as a non-invasive procedure, is investigation of choice in preoperative imaging of biliary tree[5,49,54-56]. A gadoxetic acid-enhanced MRCP in order to diagnose the biliopancreatic bile reflux and pancreatico-biliary reflux of pancreatic secretions in patients with anomalous union of the pancreatico-biliary duct has been described in the literature. Gadoxetic acid-enhanced MRCP can visualize the physiology of bile excretion, in contrast to conventional T2-weighted MRC which can visualize fluid filled space by heavily T2-weighted and fat-suppressed images. It is associated with specific properties of the gadoxetic acid which is uptaken by hepatocytes. Gadoxetic acid is excreted into the bile ducts that allows visualization of the bile ducts on hepatobiliary phase T1-weighted images[59].

PTC

Preoperative PTC allows to define the proximal extent of biliary dilatation and to use this information in preoperative resection planning[49]. According to Lipsett *et al*[53], the preoperative placement of a percutaneous ring catheter after cholangiography is useful for the surgeon during the operative procedure and can be used as a stent for the biliary reconstruction.

A technetium-99 HIDA investigation

A technetium-99 HIDA investigation is recommended to visualize the continuity BC with bile ducts. This investigation shows an initial area of photopenia within BC, with subsequent filling and then delayed emptying into the bowel[49].

CT of abdominal cavity

CT is useful in showing continuity of the cyst with the biliary tree, its relation to surrounding structures and the presence and staging of associated malignancy. It is better than USG in visualization of the intrahepatic bile ducts, distal bile duct and pancreatic head. In patients with type-ⅣA cysts and Caroli's disease, it is useful to describe the intrahepatic dilations and the extent of disease (diffuse hepatic or localized segmental involvement). It is important for a surgeon before operation, because localized type-ⅣA BC or Caroli's disease can be treated with segmental lobectomy[49]. Computed tomography cholangiography (CTC) after infusion of meglumine iodoxamate with subsequent 3-dimensional rendering has been described in the literature. Fumino *et al*[60] compared CTC cholangiography with MRCP in visualizing the pancreaticobiliary maljunction in 53 children. They noted superior visualization of the intrahepatic duct and the pancreatic system by MRCP compared with CTC. But the great advantage of CTC was its ability to produce high-quality images without respiratory artifacts in young infants in whom performing a good-quality MRCP is very difficult.

MANAGEMENT IN BC

BC require surgical intervention in order to avoid complications associated with pancreaticobiliary reflux. Management depends on the BC type. Currently, complete cyst excision with cholecystectomy followed by biliary reconstruction using a Roux-en-Y hepatico-jejunostomy is the treatment of choice[53,61,62].

Internal drainage (cystenterostomy) of BC

Currently, this method of treatment has been abandoned due to a high risk of morbidity and malignancy up to 50% following internal biliary drainage. This surgical procedure involved incision of BC and anastomosis it to the duodenum or jejunum depending to its location. Despite relief of clinical symptoms in operated patients, it was associated with a high postoperative mortality rate due to biliary reflux. Reflux of the enteric juice into the retained cyst and biliary tract leaded to recurrent ascending cholangitis. The another complication of this procedure was biliary anastomosis stricture. The most important complication of this treatment method was malignant transformation within the wall of retained BC[62-64]. Therefore, internal drainage is currently not considered to perform and all patients previously operated in this way should be reoperated in order to totally remove the cyst[62,63,65,66].

Total excision of BC with hepaticoenterostomy

Currently, total BC excision is recommended in order to avoid above mentioned complications. Total BC excision

with hepaticoenterostomy separates the biliary tree from the pancreatic duct that ends mixing pancreatic juice with bile. In situations where the intensity of fibrosis precludes safe periductal or pericystic dissection, Lilly's technique is useful. In this technique, the most densely adherent portion of the cyst wall is retained on the hepatoduodenal ligament, removing only the less adherent portion. The mucosal lining of the retained cyst wall should be ablated by diathermy or be stripped or destroyed by abrasion and iodine or alcohol application, because 57% of the bile duct cancer in BC arises from the posterior wall of the cyst[54,63-70].

There are two possible methods of hepaticoenterostomy: hepaticoduodenostomy and Roux-Y hepaticojejunostomy. According to the literature, the success rate of Roux-Y hepaticojejunostomy is 92%, with complication rate of 7%, compared with a complication rate of 42% following hepaticoduodenostomy. Some authors have compared these two reconstructions[63]. Shimotakahara *et al*[71] did not recommend hepaticoduodenostomy for reconstruction after BC excision due to a higher number of complications (33.3%) such as bilious gastritis due to duodenogastric bile reflux and adhesive bowel obstruction and cholangitis. Contrary to this report, Mukhopadhyay *et al*[72] recommended hepaticoduodenostomy as a simple and quick procedure with preservation of normal anatomy and physiology and minimum complications.

Some technical aspects of Roux-Y hepaticojejunostomy should be discussed. According to the literature, the anastomosis should be wide in order to prevent the postoperative stricture and Roux-Y jejunal loop should be 40-50 cm long[66]. Conventionally, an adult's standard of a 40-cm loop is adopted in Roux-Y hepatojejunostomy) also in children, irrespective of patient size. Diao *et al*[73] compared two patients groups according to Roux-loop length: (1) conventional group (n = 108) where a standard 35-40 cm Roux-loop length was used regardless of the child's size, and (2) short Roux-loop group (n = 110) in which the Roux-loop length was based on the distance between hepatic hilum and umbilicus. The mean Roux-loop length in patients in the second group was significantly shorter than in the conventional group. There was no significant difference between both groups in age, operative blood loss, operative time, postoperative hospital stay, and duration of drainage. In the conventional group, 2 of (1.8%) 108 patients developed Roux-loop obstruction was observed in 1.8% patients in 1st group and did not appear in 2nd group. Mild reflux was reported in 2 patients in 1st group and 1 patient of 2nd group. Authors concluded that shorter Roux-Y loop according to patient's size can be safe and effective alternative for biliary reconstruction in children with BC.

Another technical aspect of BC surgery is management with the distal bile duct stump, after cyst excision, which is usually ligated. Diao *et al*[74] compared two patients groups in order to investigate the feasibility of selectively leaving distal stump unligated. In this study, patients were divided into two groups: (1) non-ligation group (n = 207), where the distal stump was stenotic and was left unligated, and (2) ligation group (n = 63), where the distal stump was not stenotic and was ligated. The pancreatic juice leakage rates were compared. Authors concluded that not ligating distal stump is a feasible approach in patients with the stenotic distal common bile duct. In authors' opinion, this management simplifies the operative procedure and may minimize pancreatic duct injury.

Different treatment depending on BC type

Total cyst excision with Roux-Y hepatojejunostomy has been recommended by most authors in the treatment of I and Ⅳ types of BC[62]. The risk of malignancy in type Ⅱ and Ⅲ BC is low. Therefore, complete cyst excision is not necessary. Simple excision of type Ⅱ BC is sufficient. Choledochoceles often just require endoscopic sphincterotomy in order to allow free duodenal drainage of bile and stones. Large choledochoceles should be treated surgically and excised *via* duodenostomy because of biliary, duodenal or gastric outlet obstruction. In some cases of small cysts, endoscopic excision is possible. Type ⅣA BC is treated by surgical cyst excision and a wide hilar hepaticoenterostomy, but clinical symptoms are frequently observed in patients following operation that is caused by the intrahepatic involvement of disease. If the intrahepatic involvement is localized, a segmental hepatectomy can be performed. In case of diffuse disease, a percutaneous hepaticojejunostomy, surgical or endoscopic unroofing of some intrahepatic cysts can be made. Unilobar Caroli's disease can be treated by hepatic lobectomy. Diffuse Caroli's disease with recurrent cholangitis, liver failure and cirrhosis and portal hypertension or malignant disease requires orthotopic liver transplantation[61]. There are a lot reports regarding laparoscopic excision of BC with extracorporeal biliary reconstruction. Total intracorporeal biliary reconstruction has been also described in the literature. This minimally invasive treatment method is safe and effective, and it is associated with earlier postoperative feeding and discharge from hospital[75,76].

The timing of BC surgery

Early surgical treatment in order to prevent further complications is recommended. It has been observed that early diagnosis and surgical treatment was associated with a lower morbidity compared with later diagnosis and treatment. Therefore, prenatal diagnosis is such important[77,78]. Foo *et al*[77] evaluated the clinical outcomes of patients with prenatally diagnosed choledochal cysts compared with those diagnosed after birth and the optimal timing of definitive treatment. The mean age at operation for the prenatally diagnosed group was 4.4 mo. For the postnatal diagnosed group, the mean age at operation was 5.7 years. Based on analysis of 45 patients, they concluded that prenatal diagnosis of BC resulted in earlier

definitive surgery. In patients operated in older age, more adverse complications were observed[77]. Tsai *et al*[79] compared results of surgical treatment according to patients age at the time of surgery. They compared three patients groups that had been operated: < 1 year old (group Ⅰ, n = 26), 1-16 years old (group Ⅱ, n = 48), and > 16 years old (group Ⅲ, n = 33). Group I suffered significantly fewer surgical complications and less severe liver fibrosis compared with groups Ⅱ and Ⅲ. Therefore, authors recommended early surgical treatment. Germani *et al*[80] recommended the surgical treatment after 3 mo of life to increase the success according to the size of the anatomic structure. They recommended the early treatment before 3 mo of life, only in case of severe symptoms.

RESULTS OF THE SURGICAL TREATMENT OF BC

Short-term results and early complications

Surgical treatment of BC is successful in more than 90%. It is associated with a low postoperative morbidity (2.5%-27%) and mortality (0%-6%) rate according to the surgical center and patient's age. The following early complications have been reported in the literature: biliary-enteric anastomosis dehiscence with biliary fistula, intra-abdominal bile collection (biloma) or abscess and acute peritonitis, pancreatic leakage and pancreatic fistula due to injury to the pancreatic duct, acute pancreatitis, acute cholangitis, ileus caused by bowel obstruction due to intussusception or bowel kinking due to manipulation or adhesions, wound infection and wound dehiscence, gastrointestinal bleeding, hepatic failure, and multiple system organ failure[49,54,64,65,67,68,78,81-87].

Long-term results

Late postoperative complications occur in up to 25% patients and they are the following: biliary-enteric anastomosis stricture, peptic ulcer disease, cholangitis, biliary and intrahepatic stones, pancreatitis, liver failure and biliary cancer. Authors recommend to perform a wide bilio-enteric anastomosis in order to prevent its stricture[49,54,64,67,68,77,80-88]. According to the literature, 0%-6% incidence of malignancy occurs following surgery. It is caused by remnant cyst tissue or subclinical malignant disease which had not been detected before surgery. Therefore, some authors recommend intraoperative endoscopic ultrasonography and histopathological investigation of frozen sections to rule out dysplasia, hyperplasia and malignant disease. All patients with BC require long-term follow-up for bile duct cancer, using ultrasonography and laboratory investigations including liver parameters and cancer markers [carcinoembryonic antigen (CEA), CA 19-9, CA-125][49,87-89]. CA 19-9 is the mosts significant, because it is elevated in up to 85% of patients with cholangiocarcinoma. CEA is raised in about 30% of patients and CA-125 in 40%-50% of patients with cholangiocarcinoma. These markers are not specific for bile duct cancer, because their levels are also raised in other neoplasms and inflammatory diseases[90]. Lee *et al*[91] analyzed factors predicting cholangiocarcinoma in patients operated for BC. They pointed the following factors: age > 40 years, the absence of a gallstone, elevated CEA or cancer antigen 19-9 serum level, and the presence of anomalous pancreaticobiliary ductal union in univariate analysis, and an elevated cancer antigen 19-9 level in multivariate analysis. Therefore, laboratory investigations of CEA and CA 19-9 in patients following surgery for BC are useful.

Cytology of bile and bile ducts specimens taken during ERCP ora PTC by brush or needle biopsy play an additional role in cholangiocarcinoma diagnosis. Negative cytology from brushing does not exclude malignancy. Combined brush and biopsy cytology specimens increase sensitivity to 40%-70%[90].

Frequency of follow-up in patients following BC resection has not been clearly established in the literature. In my opinion, laboratory investigations including liver parameters and cancer markers (CA 19-9 and CEA) and ultrasonography should be performed as a screening every 6-12 mo.

In cases of the intrahepatic lithiasis and Caroli's disease, the use of ursodeoxycholic acid (UDCA) in the treatment has been described in the literature. Ros *et al*[92] UDCA observed that therapy with UDCA caused clinical remission, return to normal liver function, and dissolution of intrahepatic stones on USG in all patients. Therefore, litholytic therapy was indicated by authors for intrahepatic stones in Caroli's syndrome. Successful treatment of patients with the hepatilithiasis and Caroli's disease was also described by Guma *et al*[93].

In conclusion, clinical presentation and management of BC depend on the cyst type according to Todani classification. The early and proper treatment allows to avoid serious complications (including bile duct cancer) in patients with BC. The early treatment is also associated with a lower number of complications. In most cases, total cyst excision with Roux-Y hepaticojejunostomy is the treatment of choice. Surgical treatment of BC is associated with low morbidity and mortality. Patients following surgery for BC require permanent and careful postoperative observation because of possibility of biliary anastomosis stricture and biliary cancer in tissue remnant.

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FRONTIER

Quality of ulcer healing in gastrointestinal tract: Its pathophysiology and clinical relevance

Tetsuo Arakawa, Toshio Watanabe, Tetsuya Tanigawa, Kazunari Tominaga, Yasuhiro Fujiwara, Ken'ichi Morimoto

Tetsuo Arakawa, Toshio Watanabe, Tetsuya Tanigawa, Kazunari Tominaga, Yasuhiro Fujiwara, Ken'ichi Morimoto, Department of Gastroenterology, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan

Author contributions: Arakawa T performed all the managements and decision making to conduct animal experiments and clinical studies, and prepared this manuscript; Watanabe T and Tanigawa T performed most of the animal experiments; Tominaga K and Fujiwara Y performed most of the human study of peptic ulcer disease; Morimoto K performed the human study of Crohn's disease.

Correspondence to: Tetsuo Arakawa, MD, PhD, Professor, Dean and Chairman, Department of Gastroenterology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. arakawat@med.osaka-cu.ac.jp

Telephone: +81-6-66453810 Fax: +81-6-66463413
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Abstract

In this paper, we review the concept of quality of ulcer healing (QOUH) in the gastrointestinal tract and its role in the ulcer recurrence. In the past, peptic ulcer disease (PUD) has been a chronic disease with a cycle of repeated healing/remission and recurrence. The main etiological factor of PUD is *Helicobacter pylori* (*H. pylori*), which is also the cause of ulcer recurrence. However, *H. pylori*-negative ulcers are present in 12%-20% of patients; they also recur and are on occasion intractable. QOUH focuses on the fact that mucosal and submucosal structures within ulcer scars are incompletely regenerated. Within the scars of healed ulcers, regenerated tissue is immature and with distorted architecture, suggesting poor QOUH. The abnormalities in mucosal regeneration can be the basis for ulcer recurrence. Our studies have shown that persistence of macrophages in the regenerated area plays a key role in ulcer recurrence. Our studies in a rat model of ulcer recurrence have indicated that proinflammatory cytokines trigger activation of macrophages, which in turn produce increased amounts of cytokines and chemokines, which attract neutrophils to the regenerated area. Neutrophils release proteolytic enzymes that destroy the tissue, resulting in ulcer recurrence. Another important factor in poor QOUH can be deficiency of endogenous prostaglandins and a deficiency and/or an imbalance of endogenous growth factors. Topically active mucosal protective and antiulcer drugs promote high QOUH and reduce inflammatory cell infiltration in the ulcer scar. In addition to PUD, the concept of QOUH is likely applicable to inflammatory bowel diseases including Crohn's disease and ulcerative colitis.

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Key words: Quality of ulcer healing; Peptic ulcer disease; Recurrence; Prostaglandin; Cytokines; Growth factors

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BIOGRAPHY

Professor Tetsuo Arakawa (Figure 1) received his MD

and PhD degrees from Osaka City University Graduate Medical School, Japan. He was Visiting Research Scholar and Professor at the University of California, Irvine (1990) and the University of Arizona, Tucson, AZ, United States (2000). He is currently Dean and Chairman, Department of Gastroenterology, Osaka City University Graduate School of Medicine, Osaka, Japan. His Curriculum Vitae lists 404 original articles, 421 review articles, 275 book chapters, 24 edited books, prestigious academic awards and honors, and numerous presentations at national and international meetings, including American Gastroenterological Association (AGA), American College of Gastroenterology (ACG), Pasteur Institute and others (over 300 presentations). He has been President/Governor: Japanese Gastroenterological Association, Japanese Society of Gastrointestinal Endoscopy, Japanese Society of Ulcer Research, International Conference on Ulcer Research, Japanese Society of Neurogastroenterology, Japanese Society of Gastroenterological Immunology, and Japanese Society of Capsule Endoscopy chaired numerous sessions during American Gastroenterological Association Meetings and is member of 10 national/international societies, including Fellow of AGA and Fellow of ACG. His research interests cover numerous areas of gastroenterology, endoscopy and pathophysiology; he significantly contributed to the concept of cytoprotection, molecular mechanisms of gastric ulcer healing, mechanisms of anti-ulcer drugs action and translation of basic research to clinical application.



Figure 1 Tetsuo Arakawa, MD, PhD, Dean, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan.

INTRODUCTION

Prior to the introduction of *Helicobacter pylori* (*H. pylori*) eradication treatment, peptic ulcer disease (PUD) had been a chronic disease affecting 5%-10% of the population with a cycle of repeated healing/remission and recurrence. The treatment aimed at eradication of *H. pylori* has changed the life cycle of this disease and the prevalence rate has dramatically decreased. However, the dictum "no *H. pylori*, no ulcer" is over-rated[1]. *H. pylori*-negative PUD exists, similar to PUD in nonusers of nonsteroidal anti-inflammatory drugs (NSAIDs). This PUD is referred to as non-*H. pylori*, non-NSAIDs ulcer or idiopathic PUD (IPUD)[2]. A prevalence rate of IPUD has been reported as 12%-20%, suggesting that IPUD is not rare[2]. A recent report has shown the increase in the prevalence of IPUD[3], however this remains controversial[4].

The recurrence rate of IPUD is higher than *H. pylori* and/or NSAID-associated PUD[5] and their management is more difficult and more costly compared to other ulcers[5]. The recurrence rate of ulcer may be related to quality of ulcer healing (QOUH). The pathogenesis of poor QOUH may be the underlying cause of the refractoriness and ulcer recurrence. In addition to PUD, the concept of QOUH may be applicable to inflammatory bowel disease (IBD), which is also a chronic and recurrent ulcer disease. It is likely that in order to obtain a permanent ulcer healing and remission, the high QOUH is necessary for any type of ulcer disease.

MYSTERY OF ULCER RECURRENCE: PAST HISTORY OF PATHOPHYSIOLOGY

Ulcer recurrence has long been thought to be an unavoidable feature of PUD, and therefore, maintenance treatment has been necessary to prevent recurrence. *H. pylori* is a major cause of both PUD and its recurrence. The bacteria are, however, not the only cause of ulcer recurrence, and still there are a certain number of patients with PUD not related to *H. pylori* and NSAIDs, referred to as IPUD[2,4]. To prevent recurrence of IPUD, the investigations examining the pathophysiology of ulcer recurrence are important.

Oi *et al*[6] have proposed the double-regulation theory to explain why PUD favors the gastric angle. They examined many stomachs with ulcers that had been operated upon, and found that the ulcer site was usually at the interphase of the distal side of the glandular borderline (mucosal rule) and line of distortion of movement (muscular rule). This may be the point of combination of the mechanical tension and exposure to high concentrations of acid in the pyloric gland area. The latter mucosa has a weaker resistance to acid compared to the fundic gland area. This theory may also explain why the ulcer often recurs at the same or neighboring site of a previous ulcer. This theory, however, does not fit with duodenal or gastric ulcer at other sites. Therefore, it is important to determine how the ulcer recurs.

Pan *et al*[7] have reported that the recurrence of healed duodenal ulcer depends on the histological maturity of regenerated mucosa. They have indicated that the cause of ulcer recurrence may be related to the abnormalities of the scar of healed ulcer, leading to the concept of QOUH[7]. Takagi *et al*[8] have developed a chronic gastric ulcer model in rats by topical application of acetic acid from the serosal side of the gastric glandular borderline. This model closely mimics human PUD both in histol-

Table 1 Definition and criteria of quality of ulcer healing

Definition:
QOUH represents histological maturity of healed peptic ulcer; Evaluation of QOUH should be done to assess functional and endoscopic maturity additionally to histological maturity; For a clinical use an endoscopic evaluation of maturity should be the main method
Criteria:
1: Endoscopic evaluation of maturity
Dye-contrast method, magnified endoscopy, endoscopic ultrasonography
2: Histological maturity
Regenerated mucosa: thickness, width, morphological abnormalities of glands, inflammatory cell infiltration
Granulation tissue: angiogenesis, fibroblasts and fibrosis, regeneration of muscularis mucosae, inflammatory cell infiltration
3: Functional evaluation of maturity
Microcirculation, production of mucin, prostanoids, growth factors, cell proliferation, receptor expression, adhesion molecules

Arakawa *et al*[12]. QOUH: Quality of ulcer healing.

ogy and life cycle of ulcer recurrence. Tarnawski *et al*[9] have demonstrated in this rat model that the subepithelial mucosa of macroscopically healed gastric ulcer displays disorganized restoration of glandular and vascular structures and remains histologically and ultrastructurally abnormal. They have concluded that these abnormalities may interfere with oxygenation, nutrient supply, and with mucosal resistance and defense, and therefore could be the basis for ulcer recurrence. They proposed the concept of QOUH based on an experimental ulcer model in 1990[9-11]. In a previous clinical study by our group, we proposed the concept and definition of QOUH in human gastric ulcer in a Japanese journal, which was published in an English journal 18 mo later[12] (Table 1). A flat or non-flat ulcer scar pattern has been defined with chromoendoscopy using indigo carmine, which was not demonstrated with conventional endoscopy (Figure 2). These patterns are compatible with histological maturity of regenerated mucosa of healed gastric ulcer; the flat pattern represents a good QOUH and non-flat represents poor QOUH[13] (Figure 2). The incidence of ulcer recurrence is much higher in a non-flat scar than in a flat scar pattern[13,14] (Figure 3), suggesting that the endoscopically assessed QOUH may detect scars that are predisposed to future ulcer recurrence.

ULCER HEALING AND PROSTAGLANDINS

Prostaglandins (PGs) and the discovery of the phenomenon of cytoprotection by Robert *et al*[15] sparked an enormous interest in the critical role of PGs in mucosal defense and ulcer healing. In humans, "artificial" ulcers in the gastrointestinal tract produced by endoscopic mucosal resection for neoplasm heal rapidly and do not recur. We have shown that the surrounding mucosa of such artificial ulcers synthesizes increased amount of prostaglandin E2 (PGE2) and prostacyclin[16], which may have a crucial role in producing high QOUH. This observation has led to our contention that PUD may be a PG-deficiency disease[17] and that PG deficiency may cause poor QOUH. We have demonstrated that low-dose indomethacin causing PG-insufficiency forms an experimental chronic gastric ulcer with poor QOUH, and that exogenous PGE2 reverses poor QOUH as assessed by recurrence rate after healing, and reduces inflammatory cell infiltration of the ulcer scar[18] (Figure 4). Therefore, PG derivatives and/or PG-inducing drugs such as mucosal protective compounds may promote high QOUH[19,20].

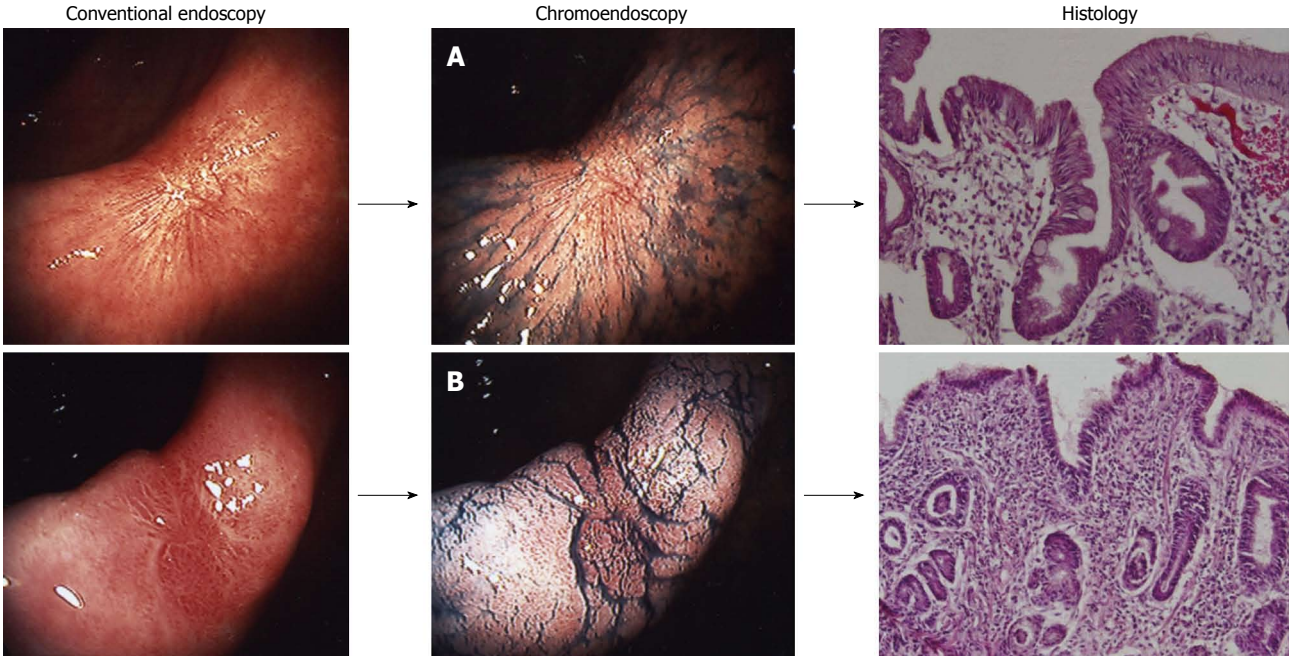
Another important factor in poor QOUH could be a deficiency and/or an imbalance of endogenous growth factors. Jones *et al*[21] have demonstrated that local gene therapy with vascular endothelial growth factor (VEGF) and angiopoietin 1 (Ang1), with limited duration of target gene expression, significantly accelerates experimental gastric ulcer healing in rats. Co-injection of both plasmids encoding rhVEGF 165 and rhAng1 resulted in formation of more mature vessels and more complete restoration of gastric glandular structures within the ulcer scar, reflecting better QOUH. Inhibition of accelerated healing by a neutralizing anti-VEGF antibody indicates an essential role for VEGF and enhanced angiogenesis in ulcer healing.

PATHOPHYSIOLOGY OF QOUH

Histological evaluation of scars of healed gastric ulcers shows increased infiltration of regenerated tissue with numerous neutrophils and macrophages in a non-flat scar compared to a flat scar (Figure 5). The persistence of chronic inflammation may be reflected by the finding of a hypoechoic area beneath the ulcer scar with a non-flat pattern, assessed by endoscopic ultrasonography (Figure 6). The number of macrophages infiltrating scar tissue is five times higher than neutrophils in a non-flat scar, suggesting that these macrophages may play a key role in pathophysiology of QOUH, and hence future ulcer recurrence. These macrophages produce increased amounts of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and monocyte chemotactic protein (MCP)-1 (Figure 7). The proinflammatory cytokines, IL-1β and TNF-α further activate and stimulate macrophages, thus constituting a self-perpetuating circuit. The increased stimulation of production of these cytokines induced by NSAIDs, stress, or *H. pylori* may cause these macrophages to increase cytokine production and/or release, leading in turn to attraction and accumulation of neutrophils. Neutrophils by releasing proteases and active oxygen species damage the scar tissue and induce ulcer recurrence.

MECHANISMS UNDERLYING ULCER RECURRENCE

As mentioned previously, Okabe's rat model of chronic ulcer mimics human peptic ulcer not only morphologi-



Endoscopic pattern	*n*	Regenerated gland index	*P*-value
Flat	10	48.0 ± 2.8	
Non-flat	5	40.2 ± 2.6	< 0.05

Figure 2 Assessment of endoscopic quality of ulcer healing. Flat pattern (A) and non-flat pattern (B) are identified by chromoendoscopy, but not by conventional endoscopy. Flat pattern reflects good quality of healing compared to nodular pattern as assessed by histological maturity (Arakawa *et al*[12]).

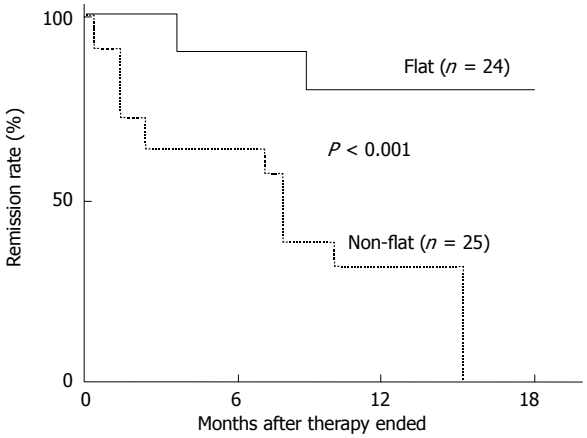


Figure 3 Cumulative remission rates of healed gastric ulcers with flat and non-flat pattern. The incidence of ulcer recurrence is much higher in the ulcer scar with a non-flat than flat pattern (Nebiki *et al*[14]).

cally and histologically but also with regard to the life cycle of spontaneous recurrence. Numerous neutrophils and macrophages persist in and beneath the regenerated epithelium even after ulcer healing. This persistent chronic inflammation may have a key role in causing future ulcer recurrence. Watanabe *et al*[22] have demonstrated that inflammatory cytokines, IL-1β and TNF-α, administered systemically in rats with macroscopically healed gastric ulcer, cause ulcer recurrence at the site of the previous ulcer (Figure 8). In this model of gastric ulcer recurrence that we reported in 1997, we found increased expression of adhesion molecules, intercellular adhesion molecule (ICAM)-1 in endothelial cells and leukocytic β_2 integrins, lymphocyte function-associated antigen (LFA-1; CD11a/CD18) and Mac-1 (CD11b/CD18) in leukocytes, and also of cytokines, IL1β and TNF-α[22], and chemokine, MCP-1[23]. This increase occurred in the regenerated tissue of the healed ulcer site (scar) 12 h after injection of an inflammatory cytokine, and was followed by massive infiltration of macrophages and neutrophils, ultimately resulting in ulcer recurrence. Antineutrophil antiserum prevents ulcer recurrence in this model, suggesting that neutrophils (producing noxious protease and active oxygen species) are the final mediator of tissue injury[22]. This hypothesis is further supported by the demonstration that antibodies against adhesion molecules (ICAM-1 and LFA-1) also inhibit ulcer recurrence[22]. These molecules regulate migration of neutrophils from arterioles into the interstitial space. Antibody against MCP-1 prevents gastric ulcer recurrence in this model[23], suggesting that the overexpression of MCP-1 in resident macrophages accumulated in the interstitial space of the ulcer scar is a first step in the mechanism of ulcer recurrence, because neutrophils and macrophages infiltrate the interstitial space of the ulcer scar only after overexpression of MCP-1[24]. Proton-pump inhibitors (PPIs) prevent gastric ulcer recurrence caused by injection of IL1β[24] and administration of

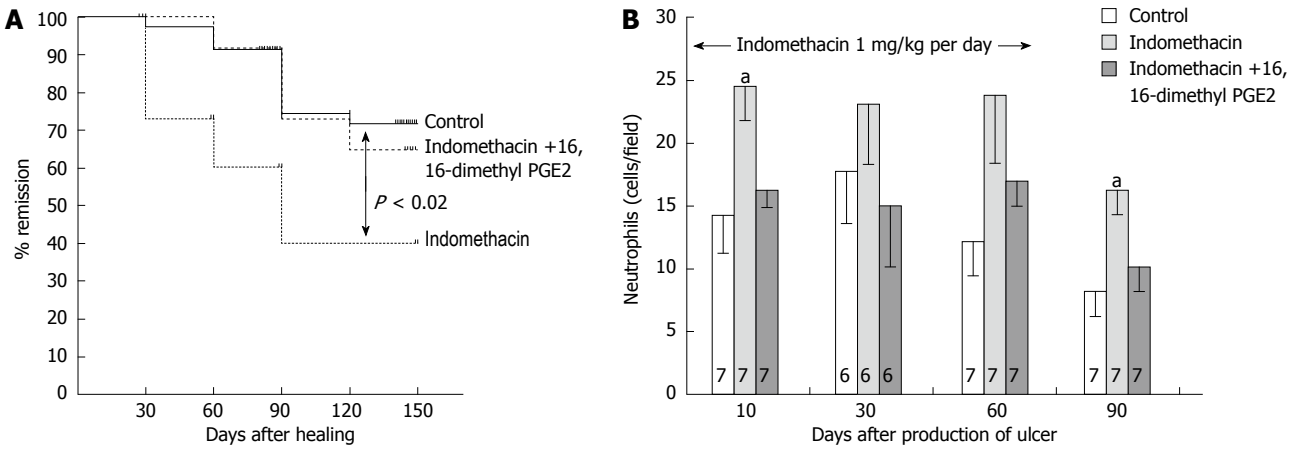


Figure 4 Effect of low-dose indomethacin given during initial period of healing process of gastric ulcer induced by application of acetic acid in rats on cumulative remission rate after healing and on neutrophil infiltration of the interstitial space of the ulcer scar. Indomethacin treatment enhances neutrophil infiltration and this phenomenon persists even after cessation of this drug. Concomitant administration of 16, 16-dimethyl prostaglandin E2 (PGE2) with indomethacin reverses these effects by indomethacin. A: Cumulative remission rate after healing; B: Infiltration with polymorphonuclear cells during and after healing. Control; indomethacin; indomethacin + 16, 16-dimethyl prostaglandin E_2 (Arakawa *et al*[18]). $^aP < 0.05$ *vs* control group.

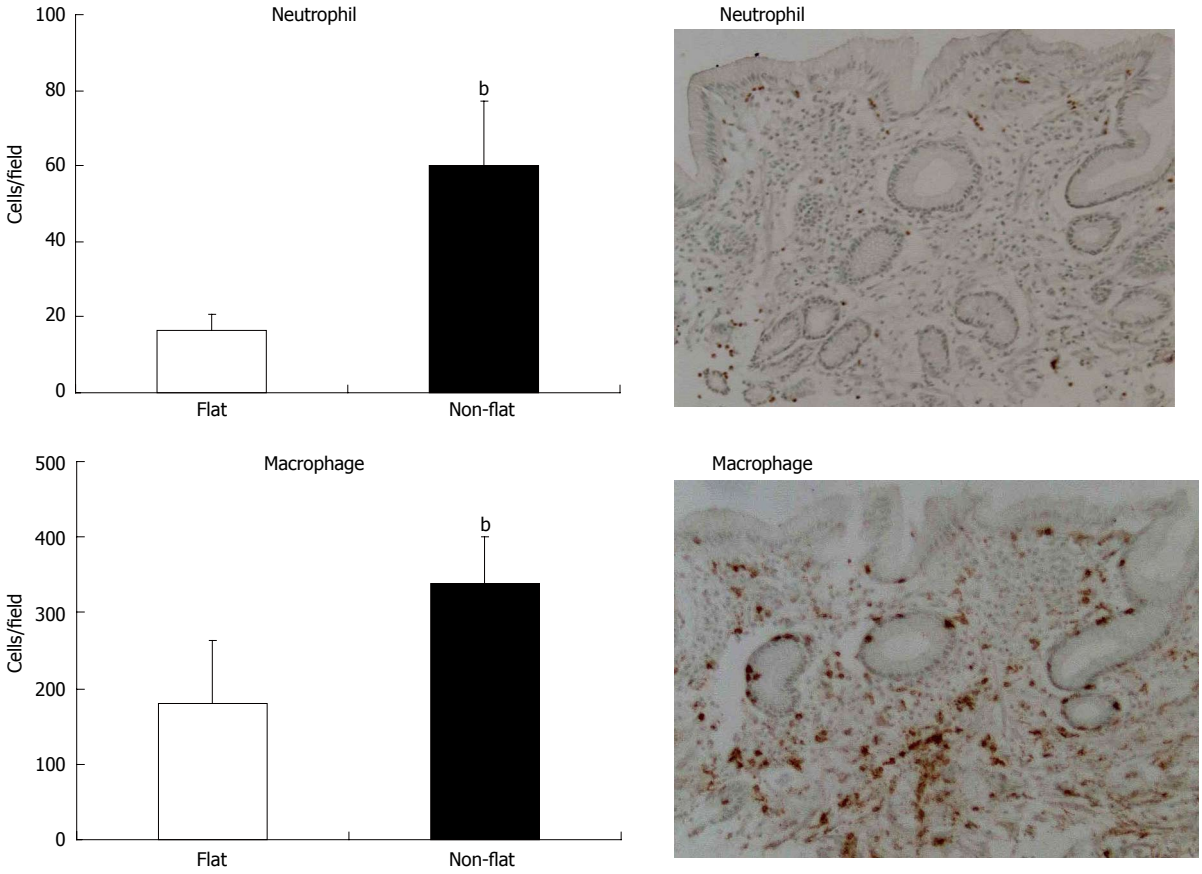
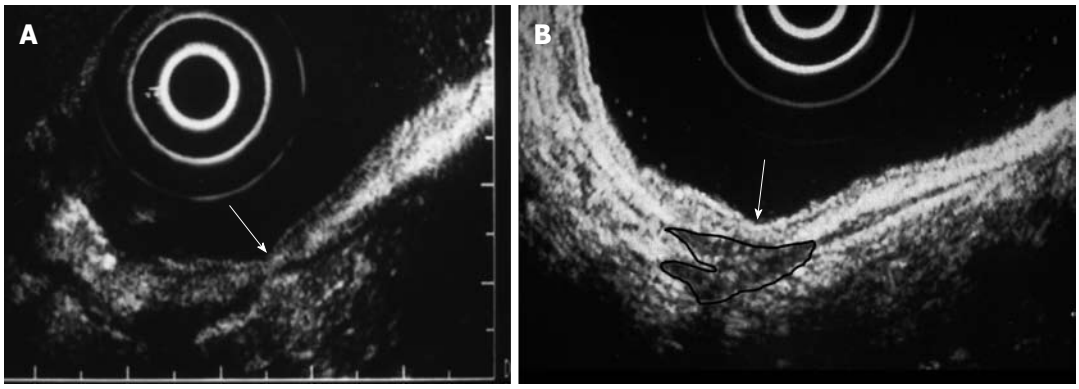


Figure 5 Number of inflammatory cells infiltrating interstitial space of ulcer scar with flat or non-flat pattern assessed by chromoendoscopy in patients with macroscopically healed gastric ulcer. The number of neutrophils and macrophages is much higher in non-flat than in flat pattern. The number of macrophages is increased five times *vs* neutrophils in non-flat scar. $n = 6$, $^bP < 0.01$ *vs* flat group.

exogenous acid reverses the protective action of PPIs on ulcer recurrence, suggesting that presence of acid is necessary to induce ulcer recurrence[24]. Based on these studies, we propose a working hypothesis of gastric ulcer recurrence presented in Figure 9[25].

EFFECT OF ANTIULCER DRUGS ON QOUH

The histological maturity of regenerated mucosa is poor after treatment with H_2-receptor antagonists[26]. This



	Flat	Non-flat	*P*-value
Internal hypoechoic area (+/-)	3/21	16/9	0.0003

Figure 6 Assessment of interstitial space beneath gastric ulcer scar with endoscopic ultrasonography. Hypoechoic area (circle of black line) is clearly seen in non-flat scar (B), but not in flat scar (A). Arrow shows a point of ulcer scar (Nebiki *et al*[14]).

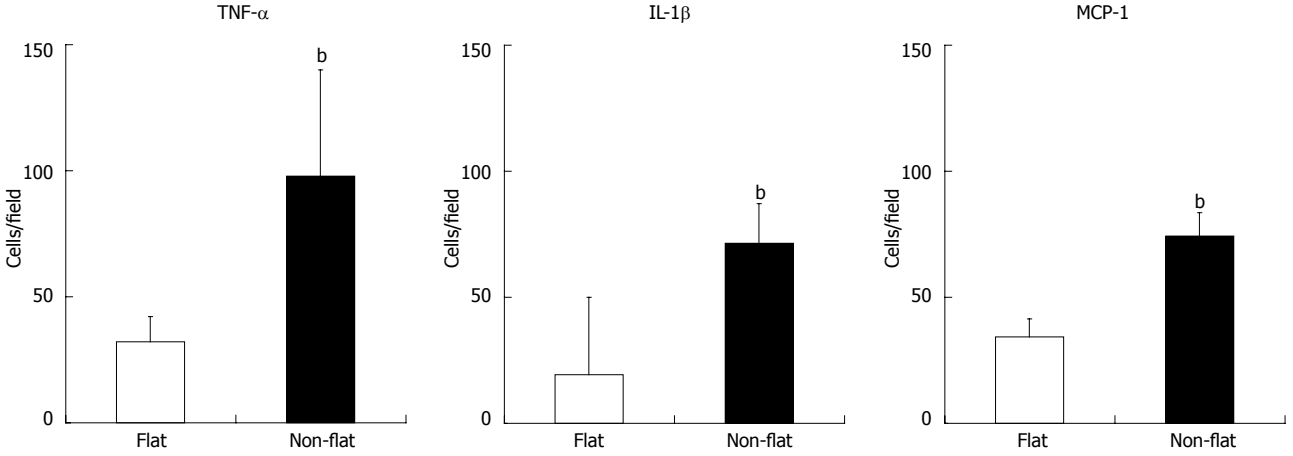


Figure 7 Number of tumor necrosis factor-α-, interleukin-1β-, and monocyte chemotactic protein-1-producing cells in interstitial space of ulcer scar site in patients with gastric ulcer whose ulcer healed with flat *vs* non-flat pattern assessed by chromoendoscopy. $n = 6$, $^{b}P < 0.01$ *vs* flat group. TNF-α: Tumor necrosis factor α; IL-1β: Interleukin-1β; MCP-1: Monocyte chemotactic protein-1.

may be related to their action to decrease mucosal PG levels[27]. Rebamipide, a mucosal-protective drug that stimulates synthesis of PGs, accelerates ulcer healing and reduces relapse of acetic-acid-induced gastric ulcer in rats. In contrast, ulcer relapse is not prevented by cimetidine[26]. Similar results regarding improving QOUH by rebamipide have been reported by Qi *et al*[28]. In a clinical setting rebamipide has been shown to improve QOUH of human gastric ulcers, as assessed by chromoendoscopy. Moreover, the ulcer recurrence rate after healing with rebamipide was similar to that found in gastric ulcer patients after *H. pylori* eradication with dual therapy[29]. Rebamipide has been shown to exert anti-inflammatory action, accelerate gastric ulcer healing, and promote high QOUH in both experimental and clinical studies[30,31].

Regarding the action of other drugs related to QOUH, antacid hydrotalcite provides better restoration of glandular structures in the gastric ulcer scar in rats compared to omeprazole[32]. In clinical studies, initial treatment with sucralfate is superior to cimetidine in decreasing recurrence rate of healed duodenal ulcer during maintenance therapy with cimetidine[33]. Direct evidence of promoting better QOUH, as assessed by chromoendoscopy, has been reported with lafutidine in a placebo-controlled trial, and ranitidine compared to famotidine in patients with gastric ulcer[34,35]. Lafutidine is a novel H_2-receptor antagonist developed in Japan. It has the unique property of stimulating capsaicin-sensitive nerves, producing NO, a cytoprotective mediator.

RECENT ADVANCES IN EVALUATION OF QOUH

Recent preliminary findings with magnifying endoscopy and narrow band imaging (NBI) may be useful for a more precise evaluation of QOUH. Magnifying endoscopy shows a fine regular glandular pattern in flat scar

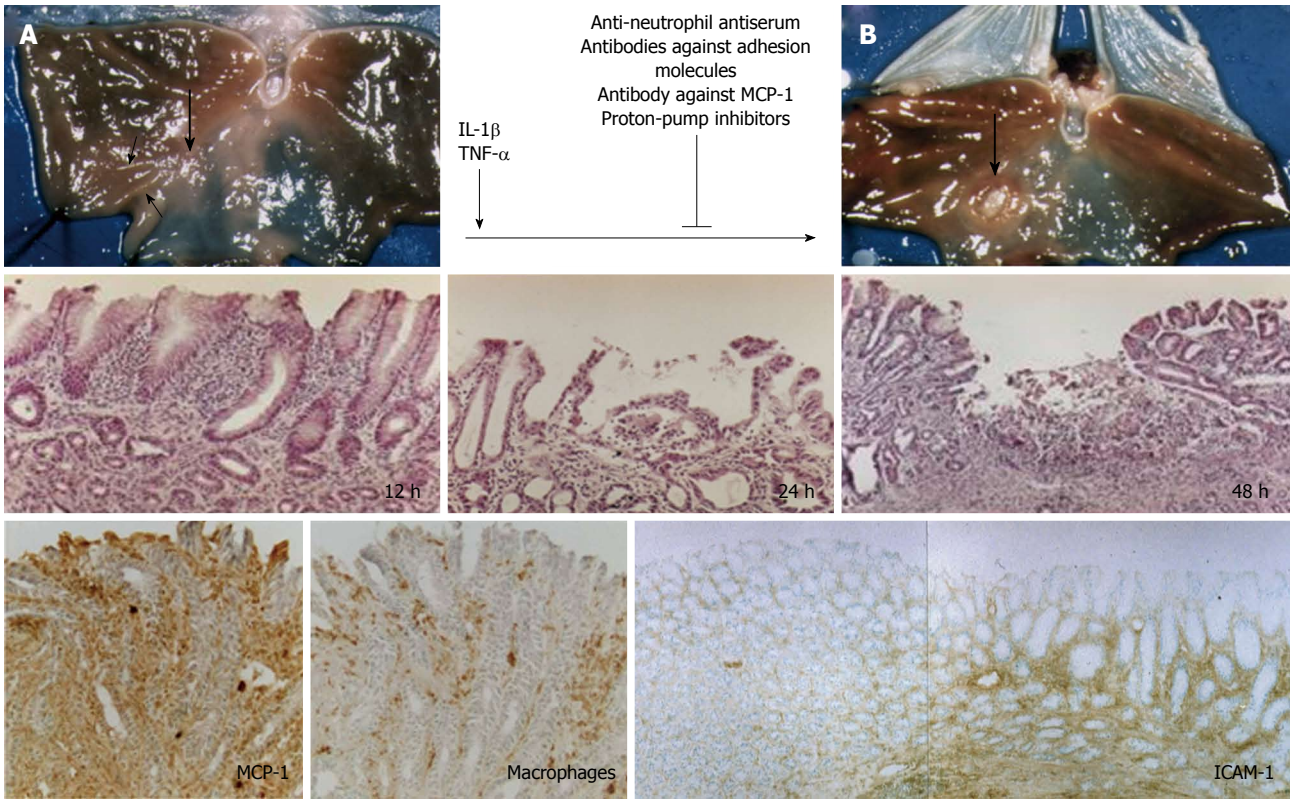


Figure 8 Original rat model of gastric ulcer recurrence. A: Ulcer scar of acetic-acid-induced gastric ulcer healed spontaneously (big arrow) with fold convergence (small arrows); B: Recurrence of ulcer with white coat at the same site as previous ulcer (big arrow). Interleukin-1β (IL-1β) or tumor necrosis factor α (TNF-α) administered systemically causes recurrence of ulcer macroscopically and histologically. Antineutrophil antiserum, antibodies against adhesion molecules, antibody against monocyte chemotactic protein-1 (MCP-1), or proton-pump inhibitors prevent recurrence. MCP-1 is overexpressed in macrophages in the interstitial space of the ulcer scar site at 12 h after administration of IL-1β or TNF-α. Adhesion molecules are overexpressed only at the ulcer scar site (Watanabe *et al*[22-24]). ICAM: Intercellular adhesion molecule.

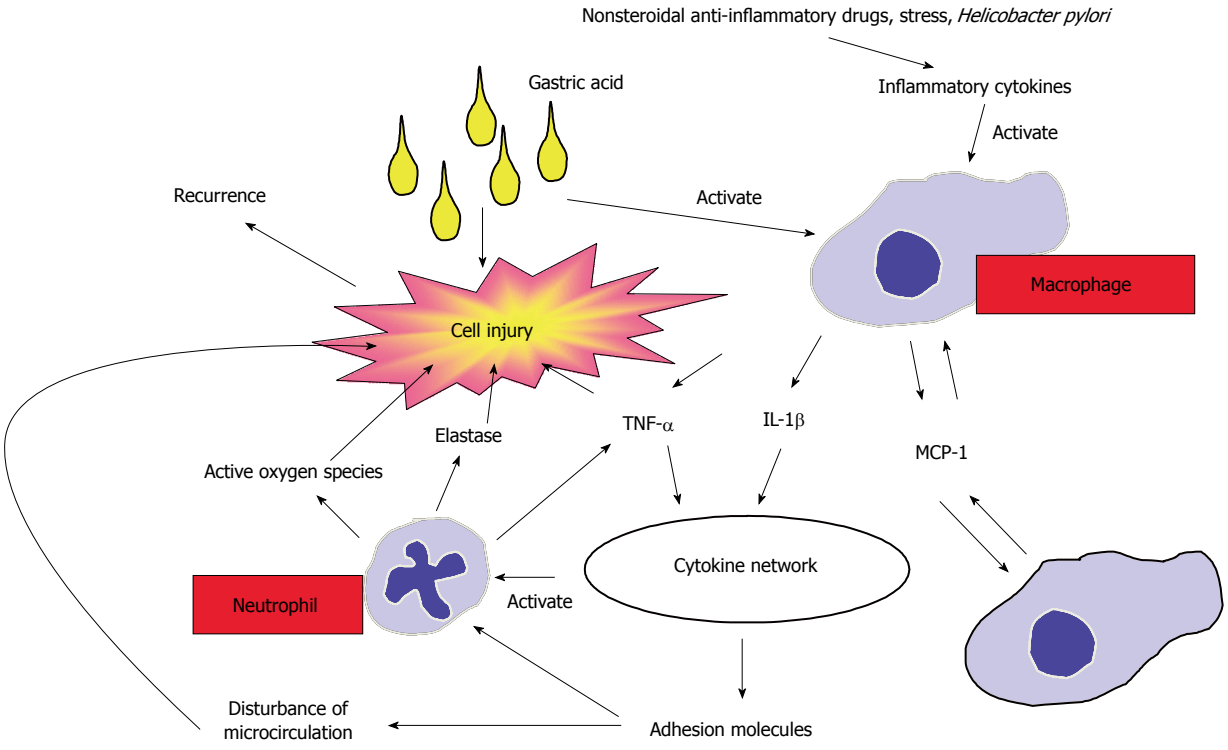


Figure 9 Proposed mechanisms of ulcer recurrence. Ulcerogenic factors such as nonsteroidal anti-inflammatory drugs, stress, and *Helicobacter pylori* stimulate the production of inflammatory cytokines, which activate macrophages under acidic conditions. The activated macrophages produce monocyte chemotactic protein-1 (MCP-1), which accumulates other macrophages. These macrophages all produce a large amount of interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), which activate the cytokine network, resulting in activation of adhesion molecules and neutrophils. Activated neutrophils migrate from arterioles to the interstitial space and injure the tissue with noxious substances such as reactive oxygen species and elastase produced by themselves together with gastric acid (Arakawa *et al*[25]).

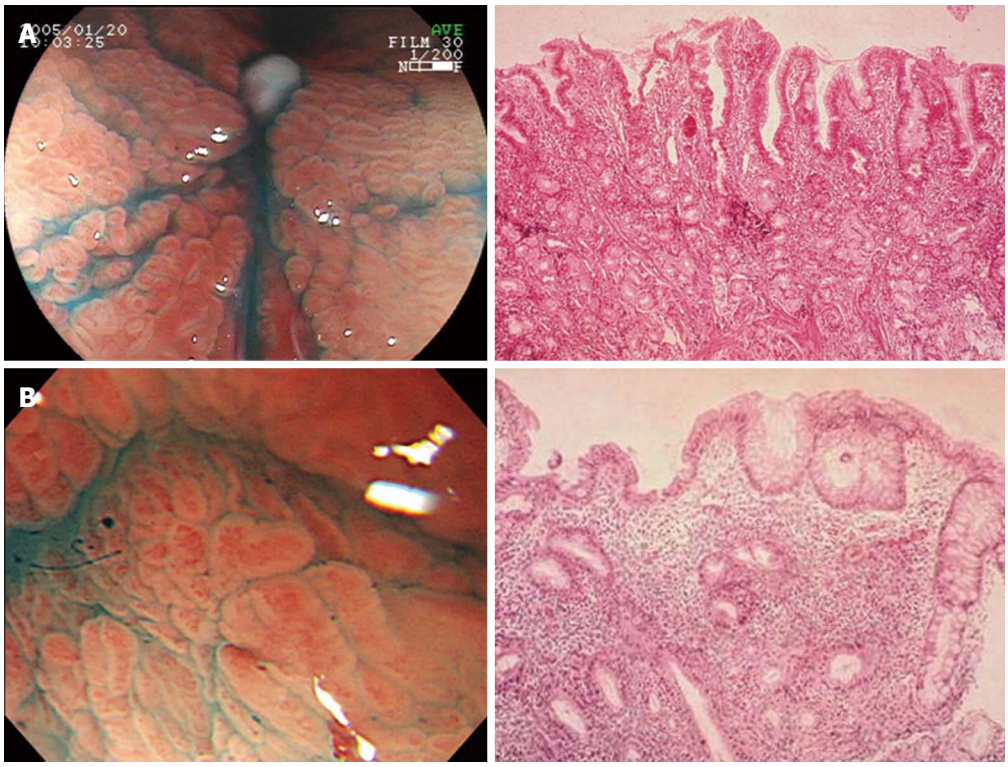


Figure 10 Magnifying chromoendoscopy shows different findings in flat scar as a fine regular pattern (A) and non-flat scar as coarse irregular pattern (B) compatible with histological findings.

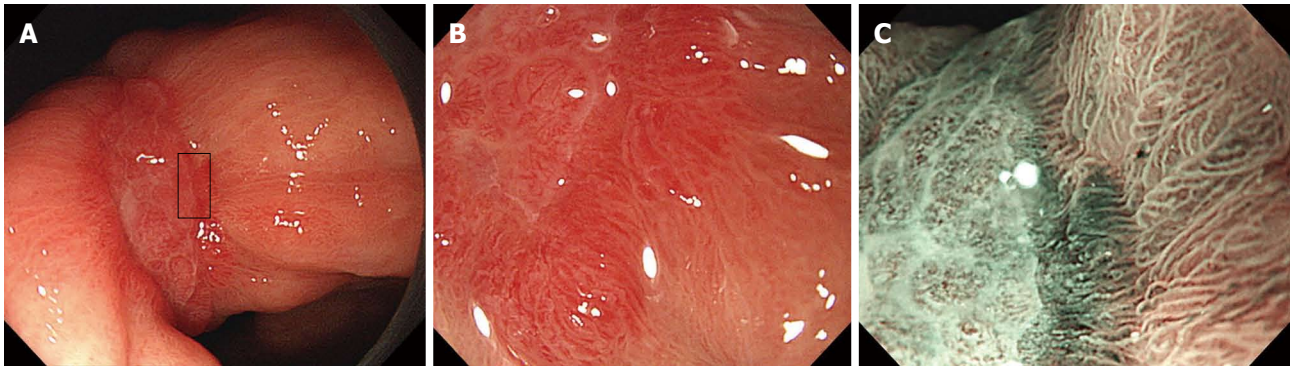


Figure 11 Narrow band imaging with magnification by endoscopy in patients with gastric ulcer shows fine vascular patterns, which may reflect precise quality of ulcer healing. A: Conventional endoscopy; B: Magnifying endoscopy; C: Magnification + narrow band imaging.

and coarse, irregular, and hyperemic glandular pattern in non-flat scar (Figure 10), suggesting that a more precise assessment is possible in the near future. In addition to this procedure, NBI may be the most promising tool to assess QOUH combined with magnification because NBI shows pattern of vessels that may reflect QOUH (Figure 11).

MUCOSAL HEALING IN CROHN'S DISEASE

Biological drugs aimed at TNF-α, such as infliximab (and other related antibodies) as well as thalidomide[36], promote ulcer healing in Crohn's disease resistant to corticosteroids. This action is completely different from the mucosal healing obtained with a PPI for gastric and/or duodenal ulcers, because a PPI inhibits only acid secretion, but may not suppress inflammation. Therefore, PPIs promote rapid healing of acid-related ulcers, but do not improve QOUH, especially in the presence of *H. pylori*. The biological drugs directly suppress inflammation, and thus they may improve QOUH. However, as long as the factor responsible for inflammation is unknown, the efficacy of such drugs may be limited.

Granulomas beneath the regenerated tissue may have an important role in cell-to-cell contact of macrophages with T lymphocytes in Crohn's disease and cause chronic inflammation, and on this background poor QOUH[37,38].

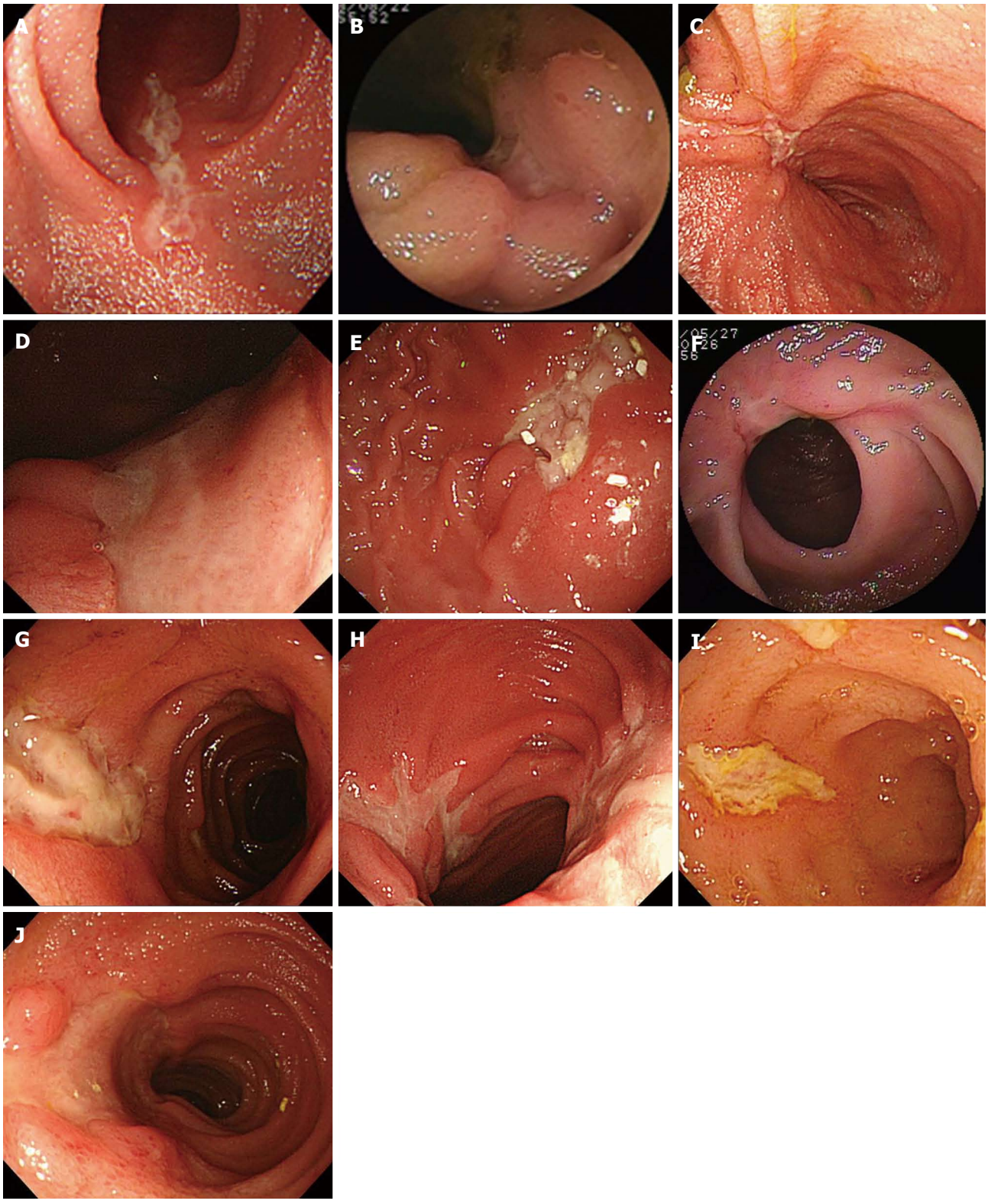


Figure 12 Morphological characteristics of active ulcer in patients with Crohn's disease. Shape of ulcer edge, flat (A) or non-flat (B); fold convergence, negative or positive (C); shape of ulcer base, flat (D) or non-flat (E); stenosis, negative or positive (F); neighboring ulcer, negative (G) or positive (H); width of ulcer, narrow (I) or wide (J).

The inflammation in Crohn's disease in based on Th1 and Th17 reaction[39-41] that is similar to *H. pylori*-associated gastritis[42,43]. Therefore, the inflammation in Crohn's disease may have similar immunopathophysiology to *H. pylori*-associated inflammation, which causes poor QOUH and underlies the ulcer recurrence. The similarity of immunological abnormality in Crohn's disease and *H. pylori*-associated gastritis may also be related to distur-

Table 2 Morphological characteristics of active ulcers in patients with Crohn's disease reflecting possible future mucosal healing after treatment with infliximab

Morphological finding		Mucosal healing		
		Good (n = 10)	Poor (n = 11)	P value[1]
Shape of ulcer edge	Flat	8	3	
	Non-flat	2	8	0.03
Fold convergence	Negative	7	3	
	Positive	3	8	0.08
Shape of ulcer base	Flat	4	3	
	Non-flat	6	8	0.65
Stenosis	Negative	8	9	
	Positive	2	2	0.99
Neighboring ulcer	Negative	9	4	
	Positive	1	7	0.02
Width of ulcer	Narrow	9	4	
	Wide	1	7	0.02

[1]Fisher's exact test. *P* value indicates the statistical difference in morphological findings between good and poor mucosal healing.

bance of the ghrelin system[44,45].

In our preliminary study of 21 patients with Crohn's disease treated with infliximab, morphological characteristics of active ulcer were defined, namely shape of ulcer edge, flat or non-flat; fold convergence, negative or positive; shape of ulcer base, flat or non-flat; stenosis, negative or positive; neighboring ulcer, negative or positive; width of ulcer, narrow or wide (Figure 12). The incidence of mucosal healing of active ulcer with a flat edge, absence of neighboring ulcer, or a narrow width of ulcer is significantly higher. A negative mucosal fold convergence tends to be more frequent than non-flat ulcer edge, positive fold convergence, positive neighboring ulcer, or wide ulcer (Table 2). The latter type of ulcer may require stronger anti-inflammatory treatment to obtain mucosal healing. These ulcers may need an endoscopic follow-up observation with assessment of mucosal healing after remission. If the ulcer does not recur, the mucosal healing may be high QOUH. Mucosal fold convergence (Figure 12C) may be one of the factors reflecting poor QOUH. Therefore, prevention of the convergence may improve QOUH of Crohn's disease. Stimulation of the local angiotensin Ⅱ system may have a key role in fibrosis, resulting in fold convergence and stricture[46], suggesting that inhibitors on this system such as angiotensin Ⅱ receptor blockers may promote high QOUH in Crohn's disease. However, to identify a causative factor of inflammation in Crohn's disease is the most essential. The responsible antigen needs to be elucidated from candidates such as yeast-like antigen[47], and others. Elimination of the possible antigen may be a powerful tool to obtain high QOUH and permanent remission.

The concept and the paradigm of QOUH may also apply to ulcerative colitis. Tarnawski and coworkers using confocal endomicroscopy and molecular imaging have demonstrated in colonic mucosa of patients with ulcerative colitis in remission impaired crypt regeneration, persistent inflammation, pathological angiogenesis and increased microvascular permeability; all reflecting impaired QOUH. They have found that the underlying mechanisms include dysregulation of survivin, aberrant activation of VEGF gene, and persistent inflammatory cell infiltration[48].

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GUIDELINES FOR CLINICAL PRACTICE

Use of the tumor necrosis factor-blockers for Crohn's disease

Alan BR Thomson, Milli Gupta, Hugh J Freeman

Alan BR Thomson, Division of Gastroenterology, Department of Medicine, University of Western Ontario, London N6A 5A5, Canada
Milli Gupta, Department of Medicine, University of Alberta, Edmonton T6G 2X8, Canada
Hugh J Freeman, Department of Medicine, University of British Columbia, Vancouver V6T 2B5, Canada
Author contributions: Thomson ABR conceived of the need for a timely, balanced, evidence-based review, reviewed the relevant literature, provided a first draft, critiqued the clinical relevance and design of trial, revised the initial draft, and communicated with the editors; Gupta M and Freeman HJ reviewed the medical literature, critiqued the papers and meta-analyses, rewrote portions of the first draft, and approved the final document.
Correspondence to: Alan BR Thomson, Adjunct Professor, Division of Gastroenterology, Department of Medicine, University of Western Ontario, London N6A 5A5, Canada. abrthomson@gmail.com
Telephone: +1-519-6858300-35300 Fax: +1-519-6633232
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Abstract

The use of anti-tumor necrosis factor-α therapy for inflammatory bowel disease represents the most important advance in the care of these patients since the publication of the National Co-operative Crohn's disease study thirty years ago. The recommendations of numerous consensus groups worldwide are now supported by a wealth of clinical trials and several meta-analyses. In general, it is suggested that tumor necrosis factor-α blockers (TNFBs) are indicated (1) for persons with moderately-severe Crohn's disease or ulcerative colitis (UC) who have failed two or more causes of glucocorticosteroids and an acceptably long cause (8 wk to 12 wk) of an immune modulator such as azathioprine or methotrexate; (2) non-responsive perianal disease; and (3) severe UC not responding to a 3-d to 5-d course of steroids. Once TNFBs have been introduced and the patient is responsive, therapy given by the IV and SC rate must be continued. It remains open to definitive evidence if concomitant immune modulators are required with TNFB maintenance therapy, and when or if TNFB may be weaned and discontinued. The supportive evidence from a single study on the role of early versus later introduction of TNFB in the course of a patient's illness needs to be confirmed. The risk/benefit profile of TNFB appears to be acceptable as long as the patient is immunized and tested for tuberculosis and viral hepatitis before the initiation of TNFB, and as long as the long-term adverse effects on the development of lymphoma and other tumors do not prone to be problematic. Because the rates of benefits to TNFB are modest from a population perspective and the cost of therapy is very high, the ultimate application of use of TNFBs will likely be established by cost/benefit studies.

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Key words: Adalimumab; Adverse effects; Certolizumab pegol; Crohn's disease; Economic evaluation; Infliximab; Secondary lack of response; Ulcerative colitis

Peer reviewer: Dr. Ana Paula Ribeiro Paiotti, Department of Pathology, Federal University of Sao Paulo, Rua Pedro de Toledo 781 5th floor, 04039-062 Sao Paulo, Brazil

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INTRODUCTION

Crohn's disease (CD) affects about 500 000 persons in North America[1], while the prevalence rates in northern Europe are 27-48 per 105[2]. A selected group of these persons will benefit from the efficacy and safety of tumor necrosis factor-α blockers (TNFBs). This use of TNFB has been reviewed by way of meta-analysis of placebo-controlled trials[3], as well as with recent consensus statements [e.g., Canadian Agency of Drugs and

Technologies in Health (CADTH, 2009)]. The overall view is that TNFBs are superior to placebo for the induction and maintenance of luminal CD or ulcerative colitis (UC). There are insufficient head-to-head studies comparing TNFB with mesalamine [5-aminosalicylic acid (5-ASA)], intermittent use of glucocorticosteroids (GCS), azathioprine (AZA), or methotrexate (MTX). In fistulizing CD, TNFBs other than infliximab (IFX) require further investigation. It remains to be established how early in the course of CD that TNFBs should be started, and what are the factors that predict which patient is most likely to require or to respond to this therapy.

MECHANISMS OF ACTION OF TNFB

There are numerous reviews on the molecule, cellular, physiological and pharmacological properties of TNFB[4-9]. Tumor necrosis factor (TNF) (aka "cachectin") has been called the body's sentinel cytokine or "fire alarm"[10], as it initiates the defence response to local injury. As a result of innate and adaptive immune responses, the level of tumor necrosis factor-α (TNFα) is increased in the serum and mucosa of patients with CD[11-13], and is a trigger for and a mediator of positive and negative feedback loops which influence the chronicity of inflammation.

TNF in the membranes (tmTNF) is a protein which undergoes proteolysis by the protease TNFα converting enzyme, forming a soluble protein, sTNF. Reverse signalling by tmTNFα may alter cellular responses to stimuli such as bacterial lipopolysaccharide[14-16]. The sTNF and the precursor tmTNF bind to the two TNF receptors, TNF receptor 1(TNFR1) (aka pSS or DC120a), and TNFR2 (aka p75 or CD120b)[1]. Binding the TNFR1IR2 to the cleft in the TNF subunits causes activation, and initiates the expression of interleukin (IL)-1, IL-6, interferon-gamma (IFN-γ), cell adhesion molecules (e.g., intracellular adhesion molecule-1), as well as a number other inflammatory molecules[17].

TNFR1 is expressed in all cells, and generates the proinflammatory properties of TNF. TNFR2 is present in immune as well as endothelial cells, and initiates the immunoregulatory properties of TNF[7]. If the TNF/TNFR1 clusters in membrane lipid rafts, it becomes antiapoptotic, whereas if TNF/TNFR1 is internalized within the cell, it becomes proapoptotic. Whether TNFR1 activates apoptosis or not depends upon accessory cell proteins, such as TNA receptor-associated factor 2 (TRAF2). TNFB affects both sTNF-mediated mechanisms, as well as tmTNF-mediated mechanisms[18]. TNFB neutralize sTNF with mean inhibitory concentrations in the nM range. TNFB alter tmTNF interactions with TNFR1 and 2, or as agonists that initiate differential induction of cytokine suppression through reverse signalling, involving signal-peptide-peptidase-like 2a (SPPL2a) and SPPL26. This leads to apoptosis, cell activation, cytokine suppression, induction of the adhesion membrane E-selectin, and resistance to endotoxin[18,19].

Recruitment of Fas-associated death domain binds and activates procaspase-8, which then activates caspase-3, and results in apoptosis. In this way, TRAF2 may lead to apoptosis. Alternatively, TRAF2 may recruit c1 activator-protein-1 (AP-1)-1 and c1AP-2. In turn, c1AP-1/-2 cause nuclear factor κ B (NF-κB) and AP-1 to block apoptosis increase anti-inflammatory proteins, and enhance cell proliferation as well as differentiation. In addition, TNF increases IL-2 receptor, *HLA-DR* antigen gene expression, and I5-A costimulator of IL-2-dependent IFN-γ production.

TNF may be immunostimulatory or immunosuppressive, depending on the genetic background of the patient, the timing and concentration of TNF[17,18], as well as depending on whether TRFR1 or TRFR2 are involved[20]. In clinical situations, TNFB do not likely function as immunosuppressants: TNFB may in fact provide immune enhancements[21], and TNFR may down-regulate some immune reactions that are activated in CD.

Reduced apoptosis in CD may cause inflammation[22], and the death domains in TNFR may induce apoptosis[23]. The tmTNF molecule has a cytoplasmic domain which can induce apoptosis by acting as a ligand for TNFRs, or as a receptor that transmits a reverse signal into the tmTNF-bearing cell[14]. In this way, TNFB may block or induce tmTNFB-mediated apoptosis. TNF-expressing cells such as monocytes, macrophages and T-cells are acted upon either by pathogen-associated molecules which express toll-like receptors (TLRs), or by NF-κB transportation factors which have been stimulated by inflammatory cytokines such as IL-1. The response to TNFB IFX in CD is determined by a single nucleotide polymorphism in the FCGR3A gene encoding for FCjR Ⅲa receptors on NK cells and macrophages[24]. TLR and NF-κB act through p38 MAPK and NF-κB to increase TNF mRNA (gene transcription) and TNF protein (translation).

Both IFX and adalimumab (ADA) induce apoptosis in peripheral blood monocytes as well as in lamina propria T-cells[25,26]. Certolizumab (CER) does not produce apoptosis *via* tmTNF, possibly because it cannot form cross-linkages with tmTNF, or because of its different epitope specificity. The fact that CER does not induce apoptosis and yet is clinically effective in CD provides evidence for mechanisms of action in addition to apoptosis being important in the clinical benefit of TNFBs.

For IFX and ADA, the presence of antibodies to the TNF reduces the serum concentrations and effectiveness of the drugs[27-29]. These antidrug antibodies form multivalent immune complexes with the TNFB, leading to their rapid clearance and therefore to reduced clinical response, as well as to the potential for the development of future infusion reactions.

It is not clear why IFX induces antinuclear, anti-ds DNA and anticardiolipid IgA or IgM antibodies. In reviewing this topic, Tracey *et al*[18] speculated that TNFB either dysregulated apoptosis and release autoimmunogenic plasma nucleosomes from the apoptotic cells, or inhibit some cytotoxic T-lymphocyte response that normally suppresses autoreactive B cells[30].

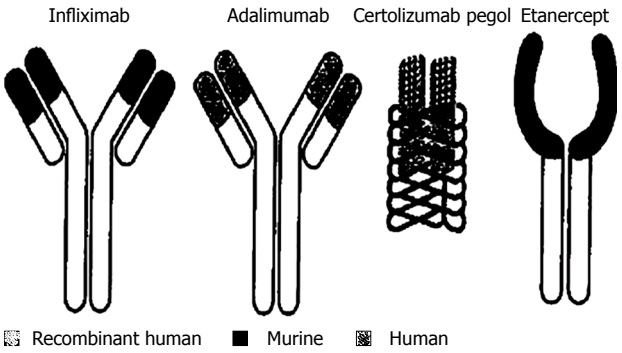


Figure 1 Comparative structures of the anti-tumor necrosis factor agents infliximab, adalimumab, certolizumab pergola and etanercept.

Control of intracellular infections such as Mycobacterium requires macrophages and T-cells in granulomas to come close to bacteria, and then wall them off. Etanercept (ETA) does not show efficacy against granulomutous diseases such as Wegener's granulomatosis and sarcoidosis[18,31]. This is unlikely the main mechanism of clinical benefit of these TNFBs in CD, since ETA is not effective in this disease. Furthermore, ETA, IFX and ADA induce apoptosis, but again ETA is not clinically active in CD. Some other pathway(s) must represent the mechanism of action of IFX, ADA, and CER in CD. IFX, ADA and CER but not ETA almost completely inhibit lipopolysaccharide-stimulated IL-IB release from monocytes, suggesting that inhibition of the production of cytokine may be important in the clinical efficacy of IFX, ADA and CER in CD[32]. Because ETA has not been shown to be clinically effective in the treatment of CD, and will not be discussed further in this paper.

The general comparative structures and individual biological properties of the TNFBs which have been approved in various countries for use in persons with CD are shown in Figure 1, and in Tables 1-3. IFX, ADA and CER are discussed in the order in which they became generally available for clinical use in CD, and the order of presentation does not imply in any way the superiority of one TNFB over another.

Etanercept is a soluble TNF receptor fusion protein (humanized IgG1 Fc fragment fused with two identical humanized p75 TNF receptors) that binds and inactivates soluble, but not membrane-bound TNFα, and although effective for the treatment of rheumatoid arthritis and other forms of inflammatory joint diseases, etanercept failed to demonstrate efficacy at similar doses in a clinical trial for CD.

Biologics other than anti-TNFα agents

Recently, natalizumab, a humanized IgG4 monoclonal antibody that antagonizes integrin heterodimers containing α4-integrin, was approved as a second-line biologic for therapy in the United States for CD patients who failed conventional therapies. Natalizumab inhibits leukocyte trafficking by preventing α4-mediated adhesion of leukocytes to adhesion molecules and transmigration of leukocytes across endothelium into inflamed mucosa[33,34]. Natalizumab is well tolerated, but is associated with an increased risk for infections, acute hypersensitivity reactions, and hepatotoxicity. The primary concern regarding natalizumab therapy has been the reactivation of latent human JC polyomavirus that can lead to a fatal central nervous system infection, progressive multifocal leukoencephalopathy, which has an estimated risk of 1:1000[35]. Therefore, natalizumab use has been restricted to monotherapy, without concomitant immune suppressants, and has a mandated special safety monitoring program and consent that has limited its patient acceptance. Nevertheless, natalizumab remains a viable option for patients who have lost a mechanistic response to anti-TNFα agents.

Table 1 The general biological properties of tumor necrosis factor blockers in clinical practice

Pro- or anti-apoptotic effects[1]
↑ Adhesion molecules on endothelial cells
↑ Epithelial permeability
↑ Antigen-stimulated B-cell proliferation and differentiation
IL-2R production[1]
↑ IL-2 dependent production of IFN-γ
↑ *HLA-DR* gene expression

[1]Represent the data are increased. IFN-γ: Intracellular interferon-γ; IL: Interleukin.

CLINICAL ASSESSMENTS REQUIRED BEFORE THE INITIATION OF TREATMENT WITH TNFBS

Absolute or relative contraindications for use of TNFBs

TNFBs are contraindicated if the CD patient has an abscess, intestinal stricture, a flare of colitis in association with an enteric infection with Clostridium difficile or cytomegalovirus, severe congestive cardiac failure (NYHA Ⅲ or Ⅳ), uncontrolled HIV disease, endemic mycosis, multiple sclerosis, or evidence of tuberculosis (TB) (positive interferon-gamma assay and/or chest X-ray prior to a four weeks course of isoniazid). TNFBs should also be used with caution and balanced consideration in CD persons with neurological disease, chronic liver disease, a history of malignancy (especially lymphoma), and in the woman who is pregnant or plans to begin parenthood (see below).

TFNBs and pregnancy: Lactation and contraception

Because the peak prevalence of inflammatory bowel disease (IBD) is between approximately 20 years to 40 years of age, for the couple where one or both partners have CD or UC, the issues of pregnancy, lactation and contraception are important. These issues need to be discussed proactively and in detail with the patient and their sexual partner.

The American Food and Drug Administration (FDA) classifies IFX and ADA as pregnancy class B agents. So,

Table 2 Individual biological properties of tumor necrosis factor blockers

IFX
High sensitivity and specificity for binding to both sTNF and tmTNF
2-3 IFX molecules bind to each TNF trimer, preventing TNF binding to cellular receptors, and reversing the actions of TNF
The TNF trimer complexes are biologically inactive and are thought to be cleared by the hepatic reticuloendothelial system
Most IFX is in the vascular compartment
No systemic accumulation
Clearance of IFN is slowed by MTX and is ATI
The VOD at steady state is independent of dose
VOD and clearance are not affected by patient age or weight. The effect of liver or kidney disease is unknown
Does not produce a generalized suppression of the body's immune system
Response to IFX is affected by CRP polymorphisms
Linear relationship between dose and serum concentration of IFX
Serum concentration is correlated with clinical response (in at least persons with rheumatoid arthritis)
Reductions in IL-10 levels also correlate with improvement in clinical activity, and FGF and improvement in perianal disease
For the 5 mg/kg dose, the median terminal half-life (t) is 10.9 d[1]
↓ Cells expressing TNF, IL-10, IFN-γ
↓ Cells staining for CD4, CD5, CD6, MMP-9
↓ TNF levels (that are ↑ in serum and diseased mucosa in CD)
↓ ICAM-1
↓ Lamina propria T-lymphocytes and peripheral blood monocytes
↓ Growth hormone resistance
↓ Markers of bone reabsorption, ↑ markers of bone formation
ADA
Forms high molecular weight complexes with human TNF (600-5000 kDa)
Linear relation between dose and serum concentration
Serum concentration is correlated with clinical response
Mean serum t is 10-20 d
Clearance is slowed by MTX, and is accelerated by ATA
Certolizumab pegol
Lacks IgG Fc domain, and thus does not fix lysed cells or complement
The Fab' component of CER contains a free cysteine residue to the hinge region, which provides site-specific attachment of the PEG to the Fab' at a site well removed from the antigen binding site
The retention time of protein conjugates in the blood is increased by pegylation, and immunogenicity is reduced. Pegylation increases in the half-life ($t_{½}$) of the antibody fragment, so dosing may be less frequent
The half-life of CER in healthy volunteers is 313 h
Site-specific pegylation of the Fab' fragment of CER is directed to a site well away from the antigen-binding region. In this way, the conjugate has the same high affinity of the TNF as the tmTNF and sTNF without the pegylation
Does not cause apoptosis of lymphocytes and monocytes, and antibodies to IFX (ATI) do not cross-react with CER
Accumulates preferentially in inflamed rather than in non-inflamed tissue
Has a greater affinity for sTNFs than do IFX or ADA
Has twice the neutralizing potency as IFX or ADA for sTNFα
Has the same neutralizing potency as IFX and ADA for tnTNFα, and occurs through p55/p75 TNFR
Anti-CER antibodies levels are low, and do not affect the efficacy of CER
Does not cross-react with ATI (antibodies to IFX)
Has a linear pharmacokinetic profile
Bioavailability of subcutaneously administered CER is almost 100%
Does not lyse cells, cause compliment-dependant cytotoxicity, antibody-dependant cell-mediated cytotoxicity, apoptosis of activated monocytes or lymphocytes or necrosis of neutrophils

[1]Represent the counts or concentration is decreased. IFX: Infliximab; TNF: Tumor necrosis factor; MTX: Methotrexate; ATI: Accelerated by antibodies to IFX; VOD: Volume of distribution; CRP: C-reactive protein; FGF: Fibroblast growth factor; IFN-γ: Intracellular interferon-γ; IL: Interleukin; MMP-9: Metalloproteinases; ADA: Adalimumab; ATA: Antibodies to ADA; ICAM-1: Intracellular adhesion molecule-1; PEG: Polyethylene glycol; CER: Certolizumab; CD: Crohn's disease; TNFR: TNF receptor.

IFX and ADA may be used during the first two trimesters of pregnancy if clinically indicated, and absolutely necessary[36-38]. There is no detailed published data on the safety of CER in pregnancy. Because clinically significant levels of IFX have been described in the serum of a baby 6 wk after delivery[39], it is recommended for the pregnant IBD mother to stop IFX in the third trimester of her pregnancy. There is no transfer of IFX to mother's breast milk, so the IFX may be restarted shortly after the child has been delivered.

Vaccination

Recommendations have been made for the regular scheduled monitoring of persons on TNFB[40]. When the diagnosis of IBD is initially established, the patient's vaccination status should be reviewed. Unfortunately, the assessment of the IBD patient for vaccine-preventable diseases is not well considered by many healthcare providers[41]. If the vaccination program is not current, then it must be updated before the use of TNFBs. Ideally, vaccinations should be updated before the use of immu-

Table 3 Comparison of *in vitro* properties of different tumor necrosis factor-α blockers in clinical practice for inflammatory bowel disease

Agent	Binds	Mediates		Increases proportion of apoptotic cells	Inhibits cytokine production	Effective in IBD patients
	TNF	CDC	ADCC			
Infliximab	Yes	Yes	Yes	Yes	Yes	Yes
Etanercept	Yes	Yes	Yes	Yes	No	No
Adalimumab	Yes	Yes	Yes	Yes	Yes	Yes
Certolizumab pegol	Yes	No	No	No	Yes	Yes

TNF: Tumor necrosis factor; ADCC: Antibody-dependent cell-mediated cytotoxicity; CDC: Complement-dependent cytotoxicity; IBD: Inflammatory bowel disease.

Table 4 Management of hepatitis B virus infection in inflammatory bowel disease patient being considered for tumor necrosis factor-α blockers therapy

Serology	Management
HBsAg+, LE ↑	Treat HBV
HbsAg+, normal LE	Vaccinate for HBV; monitor LE and HBV-DNA
HbsAg-	Vaccinate for HBV
Consider giving a combined vaccination for HAV-HBV	Measure anti-HBs-Ab titer 1 mo after third injection to determine adequacy of response; additional doses may be necessary

LE: Liver enzymes; HBV: Hepatitis B virus; HAV: Hepatitis A virus; HBsAg: Hepatitis B surface antigen.

nosuppressants such as AZA or MTX, or GCS.

Under no circumstance should the patient on TNFB be given live vaccines. To stress, these include vaccines for measles-mumps-rubella, varicella (oral), yellow fever vaccine, or oral polio vaccine. The injectable (non-oral) vaccines for polio and for typhoid are considered to be safe. Live vaccines may be given safely three weeks before or three months after stopping AZA or metronidazole (MTZ), or given at any time to persons on GCS, as long as the dose is below prednisone 20 mg/d. There are no guidelines for the ideal time to vaccinate before or after the use of higher doses of GCS.

In the IBD patient who is hepatitis B virus (HBV) positive (regardless of negative hepatitis B surface antigen, and regardless of elevated or normal liver enzyme test), the HBV must be treated before starting TNFB (Table 4).

The regular travel precautions are taken for malaria. As with all travellers, ciprofloxacin and/or metronidazole should be available to the traveler, in the event that they develop symptoms suggestive of traveller's diarrhea. It is useful if the traveler has a typed copy of their medical records, a copy of their consultant physicians' most recent letter, documentation for custom officials of their prescribed medications, purchase of traveller's insurance without exclusion for pre-existing conditions (i.e., IBD), contact names and numbers of physicians in foreign countries with IBD experience, and contact numbers for care-givers at home. IBD patients with an ileostomy should be aware of the importance of their not becoming dehydrated.

APPROVED INDICATIONS FOR TNFBS IN CD

The approved indications for the use of TNFB vary from country to country. According to the Canadian Association of Gastroenterology, as well as the Canadian Expert Drug Advisory Committee[42,43], IFX is recommended: (1) For patients with moderately to severely active CD who have continuing symptoms despite the use of conventional therapies (5-ASA, antibiotics, GCS, AZA, MTX); (2) For patients with CD who cannot tolerate conventional therapy; (3) For fistulizing CD; and (4) For pediatric CD with inadequate response to conventional therapy.

When to avoid TNFB

There are several situations when TNFB should not be used, or used after full consideration of the consequences and patient awareness and patient awareness and consent.

Narrowing of bowel and TNFB use

Narrowing of bowel and TNFB use: (1) Inflammatory-yes (no pre-stenotic dilation); and (2) Fibrostenotic-no.

Latent infection

Latent infection: (1) TB; (2) HBV; (3) HIV; and (4) Live vaccines within last 3 mo.

Active infection

Active infection: Perianal or intra-abdominal abscess must be drained.

Present malignancy or disorder

Present malignancy or disorder: Present/previous malignancy (other than non-melanoma skin cancer) or lymphoprolifer-ative disorder.

Cautious use in children

Cautious use in children: Because of 48 cases of malignancy, 88% (almost 9 out of 10) of which were while the patient was on both TNFB plus immunomodulators, and half developed lymphoma.

Efficacy of TNFBs

General considerations: There are several goals of therapy in CD (Table 5). It is important to consider a

Table 5 Goals of therapy in Crohn's disease

Induce and maintain remission off steroids
Improve patient's quality of life
Reduced risk of need for surgery or hospitalization
Reduce risk of development of adenocarcinoma/lymphoma
Maintain mucosal healing as a possible predictor or surrogate marker of better future disease outcome
Acceptable efficacy/safety balance

number of factors pertaining to the clinical trials which have been used in meta-analyses to provide therapeutic recommendations for the use of TNFBs for induction and maintenance of CD. What proportion of CD patients will have an initial non-response to IFX (primary non-response), a continued response or remission, and a secondary non-response? The definition of response and remission may vary between studies, the time of assessment of these outcomes may vary, and most studies exclude the primary non-responders at 2 wk. Also, the placebo response rates vary widely, and therefore so too does the therapeutic gain [therapeutic gain (TG); therapeutic minus placebo response]. Some patients may have a short or a long duration of CD before IFX for example was initiated used, and some trial participants were previously or concurrently on GCS, AZA, or MTX, and the dose and duration of use of GCS, AZA and MTX may have varied widely.

About a quarter of persons treated with TNFB will be primary non-responders, i.e., will not respond to an initial dose of TNFB. Because primary non-responders are often excluded from analysis, the response and remission rates in the intention-to-treat group are much lower than the per-protocol group[44]. Patients and physicians must be made aware that some of the published rates of TNFB response (about 2/3 of TNFB users) and remission (about 1/3) are in those persons who responded initially, and do not include the percentages based on all users (i.e., intention-to-treat). The overall desired outcome of TNFB therapy is prolonged steroid-free remission, mucosal healing, good quality of life, and normalization of laboratory markers of inflammation [e.g., C-reactive protein (CRP), erythrocyte sedimentation rate], an acceptably low risk/benefit ratio, and acceptable cost to ensure widespread availability.

There are different ways in which a response to therapy may be defined, and these definitions vary from study to study. In clinical trials of CD, the CD activity index (CDAI) is often used as one of the outcome measures. The CDAI is based on the patient's symptoms, the presence of an abdominal mass, anemia (reduced hematocrit), weight loss, and use of an anti-diarrheal agent. CD is arbitrarily said to be "active" if the CDAI is above 150 points. Clinical response in CD may be a decrease in CDAI of $\geqslant$ 70 points, or a decrease in CDAI of 70 to 100 points plus a 25% improvement from baseline[45]. Clinical remission might be variably defined as a CDAI of $<$ 150, or CDAI $<$ 150 plus a decrease by 50 to 100 points from baseline. Because of these variations in

Table 6 Predictors of good response to tumor necrosis factor-α blockers

Luminal
Short history of CD
Less severe disease
Isolated colonic disease
Inflammatory CD[46-49]
No previous abdominal surgery
Use shortly after ileocolonic resection
CRP > 5 mg/L (76% *vs* 46% response to TNFB), or ↑ CRP returning to normal detectable through serum TNFB (IFX) levels. CRP concentrations are inversely correlated with the rates of placebo response[50]
Non-smoking
Fistulizing
Perhaps for response with rectovaginal fistula

TNFB: Tumor necrosis factor-α blocker; CD: Crohn's disease; CRP: C-reactive protein; IFX: Infliximab.

definition, the results from one study cannot be directly compared with another study. Comparing the placebo responses gives only a very rough approximation of the comparison of the population of patients examined in two studies, because of the very large heterogeneity of CD sufferers. The only totally valid method of comparison of one TNFB to another is a head-to-head randomized controlled trial (RCT). Note that there are no head-to-head comparisons of IFX, ADA, CER. Taking the largest trials into account, and considering all of these previously mentioned cautionary points, it is suggested that there are numerically similar post-induction success rates of clinical remission and fistula closure for IFX, ADA and CER.

Specific considerations: Induction of remission, and primary non-response: Patient selection characteristics and the time in the course of the patients' disease will influence outcomes with TNFB therapy, so there may be large variations in the placebo response in one study *vs* another, and therefore gain achieved with TNFB (Table 6)[46-50]. Careful consideration must be given as when to start TNFB therapy: When to start TNFB depends upon consideration of (1) clinical characteristics (steroid-refractory and steroid-dependent); (2) previous response to treatment; (3) complications and comorbidity; (4) cost/availability; and (5) patient preference and IFX. The 2 wk induction response rates for IFX ranges from 52% to 73% (Table 7). However, the therapeutic gain for single dose IFX for induction of response and remission in CD varies with the time of assessment and dose, and is quite modest, for example, only a 10% to 22% remission rate at week 12. Quoting from the CADTH review[51], "a total of 47 citations reporting 20 RCTs and 17 observational and uncontrolled studies were included. All the RCTs had a parallel group design. Four studies were open-label, single-arm trials. Of the remaining 13 cohort studies, seven studies used a prospective design, and the remaining six studies used a retrospective design. Five cohort studies and one RCT were published as abstracts only. The remaining studies had at least one full peer-

Table 7 Therapeutic gain for single dose infliximab for induction Crohn's disease: Response and remission (%)

Assessment week	IFX (mg/kg)			PL	Therapeutic gain
	5	10	20		
Response					
2	73	52	53	17	56-35-36
4	81	50	65	17	64-33-48
12	48	29	46	12	36-17-34
Remission					
2	40	22	22	4	36-18-18
4	50	26	26	4	46-22-22
12	30	18	25	8	22-10-17

PL: Placebo; IFX: Infliximab.

reviewed publication. IFX was used as the treatment for patients with CD in eight RCTs (total number of participants 1091; range: 36-335) and 10 observational and uncontrolled studies (total number of participants 1402; range: 12-614), and for patients with UC in six RCTs (total number of participants 1477; range: 299-499) and five observational and uncontrolled studies (total number of participants 157; range: 15-36). One cohort study (10 participants) reported the use of ADA in UC. One RCT of 43 participants evaluated the effects of ENT in patients with CD. Of six which included placebo-controlled trials assessing the effectiveness of IFX in the treatment of CD, three studies used short-term regimens of IFX to induce disease remission, and three RCTs evaluated the effectiveness of long-term IFX in maintenance of remission"[51].

For the treatment of fistulizing CD, reporting on 776 patients in 10 studies, after open-label induction, maintenance trials showed a mean difference of 16% (95%CI: 8%-25%, $P < 0.001$)[3]. Thus, only 11% had induction of remission as compared to placebo, and only 8% maintained their remission for about half a year.

The TG is the treatment response minus placebo response. The TG for significant response at weeks 4, 12, 24, and 30 and 54, a response will be seen in 50% and 37%, 30, 52, and 54 is 48%, 29%, 28%, 23%, 18%, and 22% with TG for remission of 16% and 13%.

In IFX primary non-responders, there is no proven benefit in using ADA. Of those who do respond initially at 2 wk, then after 4 wk of IFX about two-thirds respond (CDAI > 70 point reduction), and about one-third are in remission (CDAI < 150 points)[52]. At 24 wk, 57% will still show a response, but will not be in remission [IFX = placebo (PL), $P = 0.31$][52,53]. At 30 wk and 54 wk, 39% and 28% were in remission[54].

Adalimumab

The two major studies evaluating ADA in CD need to be evaluated separately due to heterogeneity in terms of whether the CD patients were naive to TNFB[51]. Similar to IFX, about 30% of persons with CD given ADA will be primary non-responders. Of those who do respond initially at week 1, about 52% will have responded at 4 wk and 21% will be in remission. The secondary non-response rate to ADA is 23%-48% at 24-26 wk and 23%-59% at 56 wk. The TG (active agent-placebo response) at weeks 4, 26, and 56 was 14%, 23%-25% and 23%-24% (Table 8)[54-56].

Table 8 Comparison of tumor necrosis factor-α blockers for Crohn's disease remission and fistula closure

	IFX (mg/kg)	ADA (mg)	CER (mg)
Remission (26-30 wk)	42	43	48
Fistula closure	36[78]	32	54

CER: Certolizumab; IFX: Infliximab; ADA: Adalimumab.

The Community Economic Development Assistance Corporation recommends ADA for patients with moderate to severely active CD who are "refractory to or who experience contraindication to an adequate course of 5-ASA and corticosteroids and other immunosuppressive therapy"[42]. It is not defined how long the patient must have been on conventional therapies before declaring that they have continuing symptoms, or how minor these continuing symptoms may be before considering adding TNFB therapy. It is not clear whether patient preference represents a "contraindication" to an adequate course of conventional therapy.

The mean percentage of loss of response to ADA among primary responders was 18.2% and the annual risk was 20.3% per patient-year. The mean percentage of patients who required dose intensification among primary responders to ADA was 37% and the annual risk was 24.8% per patient-year. When considering initial responders and patients with primary non-response, the mean percentage of patients who needed an ADA dose escalation was 21.4% and the annual risk was 24.4% per patient-year. Pooled analysis showed that dose escalation permitted response to be regained in 71.4% and remission in 39.9% of patients. Predictors for loss of response or dose escalation were male gender, current/former smoker status, family history of inflammatory bowel disease, isolated colonic disease, extra-intestinal manifestations, 80/40 mg induction therapy, longer disease duration, greater baseline Crohn's Disease Activity Index (Table 9), concomitant corticosteroid use, no deep remission at week 12, low serum trough concentrations of ADA, previous IFX non-response and being previously treated with an anti-tumor necrosis factor agent.

Certolizumab pegol

The Precise 2 trial with CER in CD over 26 wk showed clear superiority to placebo in terms of clinical response and remission[57].

Meta-analysis

There have been five systematic reviews and meta-analyses published on the efficacy of TNFB in inducing remission of active luminal CD[3,58-61], as well as Cochrane review of the efficacy of natalizumab in the same clini-

Table 9 Therapeutic gain for adalimumab induction of response and remission (%)

Study	Assessment week	ADA[1]	ADA[2]	ADA[3]	PL	Therapeutic gain
Gain	Response (CDAI ↓ ≥ 100 pts)					
	1	-	-	20	12	8
	2	-	-	37	18	19
	4	-	-	38	25	13
	Remission					
	1	-	-	6	4	2
	2	-	-	21	6	15
	4	-	-	21	7	14
(Classic Ⅰ)	Response (CDAI ↓ ≥ 100 pts)					
	1	23	25	21	16	7-9-5
	2	31	37	31	15	16-22-16
	4	34	40	50	25	9-15-25
	Remission					
	1	16	13	16	7	9-6-9
	2	14	20	24	14	0-6-10
	4	18	24	36	12	6-12-24
(Classic Ⅰ)	Response (CDAI ↓ ≥ 70 pts)					
	1	-	-	6	4	2
	2	-	-	21	6	15
	4	-	-	21	7	14
	Response					
	1	37	40	32	24	13-16-8
	2	44	55	45	30	14-25-15
	4	54	59	59	37	17-22-22

[1]40 mg at week 0, 20 mg at week 2; [2]80 mg at week 0, 40 mg at week 2; [3]160 mg at week 0, 80 mg at week 2. PL: Placebo; CDAI: Crohn's disease activity index; ADA: Adalimumab.

Table 10 Meta-analysis of efficacy and safety of anti-tumor necrosis factor α antibodies *vs* placebo (4 wk to 12 wk) in inducing remission in active luminal Crohn's disease

Treatment	Outcome (RR of remission not achieving)
All biological *vs* PL	RR = 0.87; 95%CI: 0.80-0.94; NNT = 8 (95%CI: 6-17)
IFX *vs* PL[1]	RR = 0.68; 95%CI: 0.52-0.90, I^2 = 78%, P = 0.01; NNT = 4 (95%CI: 3-7)
ADA *vs* PL	RR = 0.85; 95%CI: 0.79-0.91, I^2 = 0%, P = 0.99; NNT = 7 (95%CI: 5-12.5)
CER *vs* PL	RR = 0.95; 95%CI: 0.90-1.01, I^2 = 0%, P = 0.62
Natalizumab *vs* PL	RR = 0.88; 95%CI: 0.83-0.94, I^2 = 0%, P = 0.72
Adverse effects	
	No statistically significant difference in the incidence of adverse events was detected with TNFB *vs* placebo (RR of experiencing any adverse event, including infusion or injection site reactions = 0.99; 95%CI: 0.90-1.08 With anti-α4-integrin antibodies (natalizumab) *vs* PL, significantly more patients reported headache (compared with placebo: RR = 1.23; 95%CI: 1.03 to 1.47, I^2 = 0%) With NAT there were trends towards more infusion reactions (RR = 1.41; 95%CI: 0.94 to 2.10, I^2 = 0%) and infections (RR = 1.12; 95%CI: 0.97 to 1.30, I^2 = 0%) Number needed to harm with NAT = 17 (95%CI: 9-71)

Independent data extraction was undertaken by two reviewers, and discrepancies checked and resolved intention-to-treat. Analysis and pooled data with a random effects model was used, including the reporting of the search strategy, inclusion criteria, and data extraction processes. [1]Note that when the Sonic trail was excluded from analysis, there was no statistically significant difference between IFX *vs* placebo. RR: Risk ratio; IFX: Infliximab; ADA: Adalimumab; CER: Certolizumab; PL: Placebo; CI: Confidence interval; TNFB: Tumor necrosis factor-α blockers; NAT: Natalizumab; NNT: Needed to treat.

cal setting[62]. The most recent meta-analyses on the therapeutic management of IBD have appeared in the "Red Journal" (Table 10)[61]. Funnel plot asymmetry was considered regarding evidence of publication bias and small study effects, and Cochran Q values for heterogeneity. The criteria for inclusion of studies was stringent, using rigorous and conservative methodologies and these data should be used to better guide and care for patients suffering from CD and UC.

In summary, in persons with active luminal CD who have failed treatment with first and second-line agents or who are corticosteroid dependent, for induction of remission of active luminal CD, "IFX, natalizumab, and ADA appear to have the most evidence for their use, although the latter two therapies performed only moderately in this setting"[61].

Primary non-response

The clinical definition of primary non-response is lack of improvement of clinical signs and symptoms with induction therapy. Definitions and time-frames for the assessment of response and non-response have varied amongst clinical trials for different biological agents; in the original "Targan" study of IFX in refractory CD, response was defined as a 70-point reduction in the CDAI after 4 wk[52].

To a shift in the dominant mechanism of inflammation (loss of the pharmacodynamic effect), increasing symptoms or signs not related to IBD activity (for example, irritable bowel syndrome, concomitant infection, bacterial overgrowth, and so on), or failure to wean off corticosteroids. Loss of response can also imply.

A "relative" term for patients who have shorter durations of response (for example, less than 4 wk for IFX, 1 wk for ADA, or 2 wk for certolizumab) or require dose escalation, which may become economically impractical.

In the Accent I study of IFX, which assessed the maintenance benefits of IFX, the initial response was defined as a 70-point reduction in the CDAI at 2 wk[54]. Most clinicians will consider lack of response after two consecutive infusions of IFX of at least 5 mg/kg body weight and will assess treatment failure at 4 wk[50].

Recent data suggest that patients who initially respond may, more gradually, accrue remissions over time[55].

All three currently approved anti-TNFα biological agents are administered differently and have different pharmacokinetics and dosing intervals, and a reasonable assessment of lack of response to IFX would be after 2 wk, and to ADA and certolizumab pegol would be made after completion of induction therapy at 4 wk and 6 wk.

In approximate terms, 70% of IFX-treated CD patients show a response after 2 wk of treatment. In these primary responders, the secondary non-response rate (i.e.,

Table 11 Mechanisms which are speculated to be the cause of primary non-response

Lack of response may be due to different immunoinflammatory mechanism(s)[63]
A differential role of TNFα in certain stages of disease
Individual differences in drug metabolism and elimination, drug binding in serum or tissues based on disease activity level[27,148]
The presence of innate anti-TNFα antibodies that may exhibit greater neutralizing activity in non-responders[175]
Absence of inflammation accounting for clinical symptoms
Unidentified, genetic or pharmacogenetic or serological backgrounds of individual patients[24,176-179]
Individual differences in bioavailability and pharmacokinetics, leading to inadequate concentrations of a biologic secondary to immunogenicity (neutralizing or non-neutralizing antibodies, or unmeasured or unknown antibody) or other factors that increase drug clearance (decreased circulation half -life and possible high consumption in severe disease)[27,128]

TNFα: Tumor necrosis factor-α.

Table 12 Secondary failure rates

Maintenance of response month (%)	Loss of response (%)	Loss of response per month (%)
13 (64)	36	2.8
55 (63)	37	0.7
28 (78)	22	0.8

In another long-term maintenance study, the percentage of Crohn's disease patients who maintained response declined over time: 30 wk, 83%; 54 wk, 64%; 108 wk, 45%.

Table 13 Therapeutic options for tumor necrosis factor-α blockers secondary non-response

Continue the same TNFB, but use a higher dose at the same time interval
Continue the same TNFB and the same starting dose, but use a shorter interval between doses
Re-induction therapy with another TNFB
Use another therapeutic option (e.g., tacrolimus or cyclophosphamide)
Surgery

TNFBs: Tumor necrosis factor-α blockers.

those who initially respond, but then lose their response) is 40%-50% at 24-30 wk, and 60%-63% at 52-54 wk. At one year, about 30% of the initial responders will still be responding, and about 20% will be in remission.

About 30% of persons given TNFB therapy do not initially respond (primary non-response). The explanation of this is unknown, but it may be speculated that this may be done to the use of too low a dose of IFX (the serum through concentration of TNFB generally correlates with CD response), or the inflammatory process may be independent of TNFα. Of the initial primary non-response responders, about 70% respond and 35% go into remission. Including all persons with active CD, and taking into consideration the initial non-responders, out of 100 CD patients with active disease treated with TNFBs, approximately (100 × 70% = 70, 70 × 70% = 49) will respond and (70 × 30% = 21) will go into remission. These figures are lower than historically reported clinical response and remission rate with conventional (i.e., standard-of-care) management with GCS or, AZA/MTX, however it must be stressed again that because of large differences in study populations and study designs, the clinician must be very caution about accepting comparisons in anything other than direct head-to-lead studies.

There are numerous possible reasons for lack of response (Table 11)[63].

MAINTENANCE OF REMISSION AND SECONDARY FAILURES

Once the CD patient has gone into remission, we wish to prevent relapse. In CD, we lack benefit from 5-ASA/Sulfasalazine (SASP). Antibiotic maintenance has been shown to be significantly better than placebo, but there are insufficient studies for most clinicians to use long-term antibiotics as suppressive therapy. Few clinicians are sufficiently adapt at complex statistical methodology to understand the subtle aspects of whether to believe results reported as risk ratios (RRs) or odds ratio (ORs); immunosuppressives will likely continue to be used for maintenance therapy in CD, at least in the foreseeable future.

About 43% of patients with CD require GCS again within one year of starting GCS. The lifetime need to use GCS is 44%[64]. At 14 wk of use of full therapeutic doses of GCS for moderately active CD, 84% respond and 58% are in remission. At one year 32% are in remission, 28% are still on GCS (steroid-dependent), and 40% have required surgery. It may be speculated that the use of GCS may identify a group of patients who have more aggressive disease, and in whom immunosuppression or TNFB might need to be started early in the course of the disease[65]. AZA/MTX reduce steroid-dependence and maintain disease remission, but do not reduce the need for surgery[66].

Using meta-analysis, Akobeng[67,68] demonstrated that MTX, IFX, ADA, and CER were more effective than placebo for maintaining remission in CD, with number needed to treat (NNT) of 4-6. GCS, 5-ASA, budesonide, cyclosporine, antimicrobials and probiotics did not differ from placebo for maintaining remission at 6-24 mo. Enteral nutrition (EN) was more effective than no EN. Over 50% of CD patients become steroid-dependent or have a surgical resection within one year of starting GCS[64]. The monthly rate of loss of response to TNFB is higher in the shorter than in the longer durations of follow-up, ranging from 2.8% loss of response per month in a 13-mo study, to 0.7%-0.8% loss of response per month for 28- to 55-mo long study (Table 12).

Table 14 Therapeutic gain of infliximab for Crohn's disease maintenance of response and remission (%)

Assessment week	Treatment	IFX (mg/kg)			PL	Therapeutic gain
		5	10	20		
Remission	0, 2, 6 wk					
12		75[1]	-	-	38	37[1]
24		57[1]	-	-	29	28[1]
52		40[1]	-	-	22	18[1]
Response	2, 6, 8 wk					
30		50[1]	58[1]	-	27	23-31[1]
54		37[1]	47[1]	-	15	22-32[1]
Remission	2, 6, 8 wk					
30		39[1]	39	-	21	18[1]-18
54		28[1]	38	-	14	14[1]-24
Response	q8 wk					
54	until week 54	36[1]	-	-	6	30[1]
Response	q8 wk (4 infusions)					
36		-	72[1]	-	44	28[1]
44			62[1]	-	37	25[1]
Remission	q8 wk (4 infusions)	-				
12		-	14[1]		44	-30[1]
36			60[1]		35	25[1]
44			53[1]		20	33[1]

[1]Significant difference. PL: Placebo; IFX: Infliximab.

Those who have initially responded, but then subsequently relapse, are said to be secondary non-responders. In those persons who initially respond to TNFB, and then lose the benefit (CDAI increases, representing active disease; secondary non-response), there are several therapeutic options (Table 13).

IFX

Secondary failures to IFX may be as common as 48% in the first year of anti-TNF therapy, and 66% in the second year[69]. In secondary IFX non-responders, switching to ADA results in a response in 59%-83%, and remission in 29%-50%[70-72].

Secondary non-responders may respond to an increase in IFX dose, or a decrease in dosing interval, or both[73]. In secondary non-responders to IFX, 80%-90% responded to treatment intensification with IFX[54,74]. After two and a half years after an initial response to IFX, about half (54%) of patients require dose intensification[51,75]. Other studies have confirmed the use of dose escalation[76,77]. Dose acceleration helps maintain response: for luminal disease, 90%[74], for fistulising disease, 60%[78]. At 40 wk, 65% were still responding after the last dose intensification over 52 wk[72,73]. The therapeutic gain for IFX maintenance of remission was low, ranging from 14% to 33% at one year (Table 14).

In the Accent I Study[54], dose escalation was needed for persons who initially responded but then worsened at some time during the 54 wk study in 49% of persons on episodic treatment, 30% on 5 mg/kg IFX scheduled treatment, and 26% on 10 mg/kg scheduled treatments (secondary, non-response). Response was re-established with higher doses in 90% of the 5 mg/kg and 80% of the 10 mg/kg scheduled treatment groups treated with higher doses. Most of the cross-overs to higher doses occurred at week 14. In a 40 wk study, 83% of secondary IFX non-responders did respond after the first intensification of IFX, and 65% of patients were still responding after the last intensification dose.

Patients who lost their response to IFX were switched to ADA, and then continued with it for 52 wk. Secondary non-response to ADA was seen in 25%, and therefore, patients required a dose escalation[72]. These response rates are even higher in persons treated with ADA for the first time (i.e., IFX- and ADA-naive CD patients). Two RCTs and four observational and uncontrolled studies assessed the clinical effects of ADA after a loss of response or intolerance to IFX[51]. Re-induction therapy with ADA has a 60% clinical response[79]. Similarly, the Precise Ⅱ study[57] showed a benefit of reinduction in clinical response and remission of CD with CER in those patients who had received IFX in the past .

Long-term maintenance with IFX 3 mg/kg maintains mucosal integrity and avoids CD recurrence one year after surgery[80]. Another study evaluated endoscopic recurrence of CD post-operatively and found the rates to be lower with IFX at one year *vs* placebo (9.1% *vs* 85%)[81]. However, clinical remission was not significant (80% IFX *vs* 53.8% placebo, $P = 0.38$). A recent review of the literature also shows that there is statistical benefit with IFX use within 4 wk of surgery on mucosal healing. But this effect was not statistically significant in reduction of CDAI.

Adalimumab

There are two RCTs[55,56] and four observational and uncontrolled studies[70-72,82,83] assessing the clinical effects of ADA after a loss of response or intolerance to IFX. Blinded clinical trials show that ADA is effective first-line therapy for TNFB-naive CD patients, and is an option for IFX-refractory or-intolerant persons[84]. In the Gain study[55], secondary non-responders to IFX responded better when switched to ADA than when switched to placebo (IFX→ADA *vs* IFX→PL). In an uncontrolled open-label study, the switch from IFX to ADA (IFX→ADA) was associated at week 12 with a 59% clinical response and 29% remission rate[70]. A 50% or greater decrease in the number of draining fistulas was seen in 56%, while 33% had complete absence of fistula. In a second study[82], switching from IFX to ADA (IFX→ADA) gave a partial response in 31% and a complete response in 54%. In a single-arm study[72], 50% of IFX→ADA had a clinical remission at week 52. Four weeks after switching to ADA, 83% had a clinical response and 42% were in remission[72].

In the Crohn's trial of the fully human antibody adalimumab for remission maintenance (CHARM) study[56], there was no statistically significant response to ADA in IFX non-responders (IFX→ADA) or in those who were IFX-naive. When switching from IFX to ADA[56], the response at 26 and 56 wk in the TNFB-naive patients was 47% and 42%, compared to 32% and 31% in the non-responder group. Overall the ADA

Table 15 Therapeutic gain of adalimumab maintenance of response and remission (%)

Study	Assessment week	ADA[2]	ADA[3]	PL	Therapeutic gain
	Maintenance				
CHARM	Response (CDAI ↓ ≥ 100 pts)				
	26	52[1]	52[1]	27	25[1]-25[1]
	56	41[1]	48[1]	17	24[1]-31[1]
	Response (CDAI ↓ ≥ 70 pts)				
	26	54[1]	56[1]	28	26[1]-28[1]
	52	43[1]	49[1]	18	25[1]-31[1]
(Classic Ⅱ)	Response (CDAI ↓ ≥ 100 pts)				
	24	84	94[1]	61	23-33[1]
	56	79	89[1]	56	23-33[1]
	Remission (CDAI ↓ ≥ 100 pts)				
	26	40[1]	46[1]	17	23[1]-29[1]
	56	36[1]	41[1]	12	24[1]-19[1]
	Remission (CDAI ↓ ≥ 70 pts)				
	24	95	94	83	12-11
	56	79	89	72	7-17
	Remission				
	4	95	100[1]	89	6-11[1]
	12	90[1]	89[1]	56	44[1]-33[1]
	24	84[1]	94[1]	50	34[1]-44[1]
	32	84[1]	100[1]	39	45[1]-61[1]
	48	74	94	44	30-50
	56	79	83	44	35-39

[1]Significant difference; [2]40 mg at week 0, 20 mg at week 1; [3]80 mg at week 0, 40 mg at week 2. CDAI: Crohn's disease activity index; PL: Placebo; pts: Points; ADA: Adalimumab; CHARM: Crohn's trial of the fully human antibody adalimumab for remission maintenance.

treated group did better than the placebo group. The ADA group had a higher rate of staying free of steroids for > 90 d and achieved clinical remission at week 26 (19% for 40 mg every other weeks *vs* 15% for 40 mg every week *vs* 3% for placebo, $P < 0.001$). The therapeutic gain of ADA for maintenance of remission ranged from only 25% to 39% (Table 15).

The proportion of ADA-treated patients who required dose escalation and the results of treatment were reported in two RCTs and one small before-after study[51]. In secondary non-responders to ADA, 76% responded to dose escalation and 45% went into remission[55,72,85].

There is no data available to assess the effectiveness of using IFX for secondary non-responders of ADA. In the CHARM study[55], 46% of the persons randomized to receive ADA were secondary non-responders. Of those who received dose escalation of ADA, 76% had a no-point response rate and 45% went into clinical remission. In the Classic Ⅱ study[55] (following from the Classic I study), only 5% of patients who completed the 56 wk of treatment needed dose escalation.

Certolizumab pegol

In CD patients who responded to CER induction therapy (Precise 2), and then relapsed during continuous or interrupted maintenance therapy, recapture therapy with CER was provided. The response rates at week 4 were 63%-65%, and 55%-59% at 1 year (Precise 4)[86]. Using Certolizumab pegol maintenance therapy for persons

Table 16 Therapeutic gain of certolizumab Induction of response and remission (%)

Week	Response		TG (CER-PL)
	CER	PL	
CRP < 10 mg/L			
6	37[1]	26	11[1]
26	22	12	10
Overall population			
6	35[2]	27	8[1]
26	23[2]	16	7[1]
Remission			
6	17	22	-
6 + 26	10	14	-
Maintenance with CER			
Week 26 clinical response	62[1]	34	28[1]
Overall	63[1]	36	27[1]
Group			

[1]Significant difference; [2]The secondary failure rates for CRP were 72% at 26 wk (approximately 10% per month). Precise Ⅰ study (Pegylated Antibody Fragment Evaluation in Crohn's Disease: Safety and Efficacy Ⅰ). PL: Placebo; CRP: C-reactive protein; CER: Certolizumab; TG: Therapeutic gain.

with CD, there was clinically meaningful improvement with the Inflammatory Bowel Disease Questionnaire score (60% *vs* 40%, $P < 0.001$), Short Form 36-Hem Health Survey (SF-36) physical (51% *vs* 34%, $P < 0.001$) and mental component summary responses (44% *vs* 32%, $P = 0.016$), and the proportion of persons living a normal life (21.4% *vs* 12.4%, $P = 0.019$), (57% *vs* 35%, $P = 0.001$). The therapeutic gain of CER for maintenance of remission was at 28% at week 26 (Table 16), similar to the 24 wk and 30 wk result for IFX and ADA. The therapeutic gain of CER varied little between weeks 2 and 12, and with doses of CER 100 mg, 200 mg and 400 mg (Table 17).

In the Precise 2 trial, the 26 wk therapeutic gain in response rates of therapeutic gain with CER were lower in those who had first failed IFX, than in those starting CER and never previously having been on IFX (19% *vs* 29%)[87]. This was also the case with IFX in the Precise 2 study: the response in the IFX-naive group was 69%, compared with 44% ($P < 0.001$) in the non-naive group. Thus, there may be benefit switching from IFX to ADA in the IFX-failure patient. In patients who were treated with IFX and experienced secondary failure, and then were treated with open-label CER, 62% enjoyed a response and 39% remission rate at week 6[88]. When extended to 26 wk, the response and remission rates were 40% and 37%, respectively. CER also improved work productivity during the induction and maintenance components of the Precise study[89].

Also from the Precise 2 study, 58 CD patients with draining fistulas (mostly perianal fistula) who responded initially to CER were continued on CER or placebo[90]. At week 26, 36% on CER had fistula obscure compared with 17% receiving placebo (TG, 19%). Using the definition of ≥ 50% closure at two consecutive post-baseline visits ≥ 3 wk apart, there was only a trend for achieving this end point (54% *vs* 43%, $P = 0.069$).

Table 17 Therapeutic gain of varying doses of certolizumab

Week	Response				Therapeutic gain		
	PL	CER (mg)			CER (mg)		
		100	200	400	100	200	400
2	18	33	19	28	15	1	10
4	19	37[1]	26	33	18[1]	7	11
6	18	36[1]	31	39[1]	18[1]	13	21[1]
8	22	36	29	39[1]	14	7	17[1]
10	27	44	36	44[1]	17	9	17[1]
12	23	38	24	39[1]	15	1	16[1]

[1]Significant difference. PL: Placebo; CER: Certolizumab.

Table 18 Meta-analysis of efficacy and safety of biological therapies (26 wk to 60 wk) *vs* placebo in preventing relapse of disease activity in quiescent luminal Crohn's disease

Treatment	Outcome (RR in preventing relapse)
IFX, ADA, CER	RR = 0.71 (95%CI: 0.65-0.76, I^2 = 5%, P = 0.38); NNT = 4 (95%CI: 3-5)
IFX, CER	Superior to PL
ADA	No statistically significant difference between ADA *vs* PL in preventing relapse
NAT (only one trial)	RR = 0.71; 95%CI: 0.61-0.84
Adverse events	
Any event	RR = 0.93; 95%CI: 0.84–1.03
Infusion or injection site reactions	RR = 0.64; 95%CI: 0.06-6.66; (note that these reactions were actually fewer in those on anti-TNFα therapy, but the difference was not statistically significant)

PL: Placebo; CER: Certolizumab; IFX: Infliximab; ADA: Adalimumab; TNF: Tumor necrosis factor; RR: Risk ratio; CI: Confidence interval; NAT: Natalizumab.

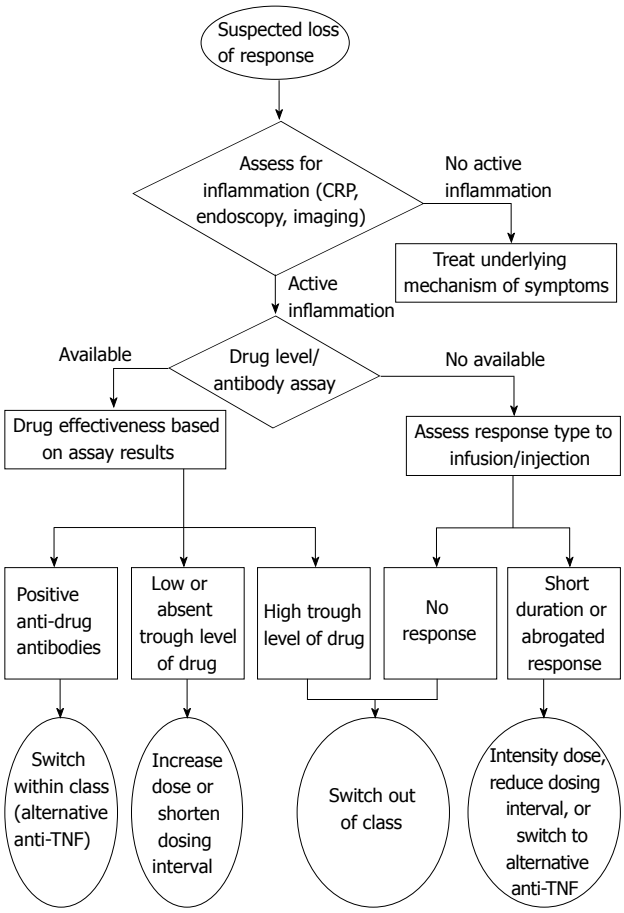


Figure 2 Suggested clinical approach to loss of response to anti-tumor necrosis factor-α therapy. CRP: C-reactive protein; TNF: Tumor necrosis factor.

The maintenance response rate was inversely related to the duration of the patient's history of CD: maintenance of response to CER was present in 90% of patients with a CD diagnosis less than one year, compared with 57% continued response in those with a CD diagnosis ⩾ 5 years[91]. Corresponding remission rates were 68% *vs* 44%. This suggests that there may be improved efficacy of TNFB maintenance therapy when initiated "early" as compared "late" in the history of the patient's disease[92].

Meta-analysis

The results of the most recent vigorous meta-analysis of biological therapies in preventing relapse of disease activity in quiescent luminal CD is shown in Table 18. These authors suggest that the most marked effect of biological therapies was in preventing relapse of luminal CD once remission had been achieved, with a NNT of only 4. This benefit was observes for both IFX and certolizumab, but not for adalimumab[61].

Secondary non-response

About a third of patients treated with anti-TNFα therapy, who meet the criteria for an initial clinical response, eventually lose response, with the magnitude depending on the duration of follow-up on maintenance therapy. Loss of response, also referred to as secondary non-response, is defined as recurrence of disease activity during maintenance therapy after achieving an appropriate induction response. In maintenance trials with IFX and ADA, significant percentages of patients required increased doses (IFX) and or decreased treatment intervals (adalimumab) in order to restore response after interval symptoms developed between dosing[54,56,78,93]. Even after scheduled maintenance therapy and dose adjustments, substantial numbers of patients will still have a poor response[44]. Of the initial responders, 65% need dose escalation, which appears to be successful in approximately 70%, and the probability for dose escalation throughout time is approximately 80% at 120 wk[94].

From the Figure 1, it is apparent that approximately 10% (56%/5 years.) of patients treated with TNFB lose their response each year. If patients who are doing well on IFX + AZA, and the IFX is stopped, 39% relapse within one year[95]. Persons who are likely to relapse when IFX is stopped are those with objective signs of continued inflammatory activity (↑CRP, Crohn's disease endoscopic index of severity ⩾ 2) or low IFX though level. Fortunately, almost all (> 95%) of those who recurred when IFX was stopped respond to IFX retreatment. Presumably a greater proportion relapse as this

interval is followed out beyond one year; it is unknown if those longer term relapsers will continue to have such a favorable response to retreatment with the originally successful TNFB.

How is this loss of response to TNFB managed? A useful suggested clinical approach to loss of response to TNFB therapy has been published (Figure 2). First determine if the patients symptoms might be due to something other than active CA. For example, exclude enteric infection (including *Clostridium difficile*) or small intestinal bacterial overgrowth, bile salt wastage, or adverse reaction to other drugs. What are the options if the CD is active[96]? The initially chosen TNFB is increased in dose, decreased in dosing interval, or both! If there is no response, switch to another TNFB[96].

Suggested clinical approach to loss of response to TNFB (Figure 2), to quote the approach suggested by Yanai *et al*[63]: (1) Confirm that intestinal inflammation is the cause of the "loss of response"; (2) Measure IFX levels at 4 wk after an infusion (therapeutic $\geqslant$ 12 μg/mL); (3) Measure antibodies to IFX (ATI) measurements not available for ADA or CER. The Mayo Clinic group has demonstrated the utility of combining IFX levels and determination of ATI in the clinical management of patients who lose response to IFX[97]; (4) "Patients who lose response to a first agent are more likely to lose response to a second and, similarly, those with toxicity to a first agent are more likely to develop toxicity from a second anti-TNFα agent. This diminishing outcome may, in part, be due to a class effect"; (5) "Patients who lose response to IFX associated with anti-IFX antibodies respond well to switching to adalimumab or certolizumab pegol"; (6) "In the majority of patients, response can be restored by dose and interval adjustments"[44,94]; (7) "Switching between biologics is an important strategy for patients who lose response because of immunogenicity followed by rapid drug clearance or when a significant alternative pathogenic role is suspected. Switching can be within class or with an alternative class"; and (8) "Efficacy of successive agents is decreased compared with the primary agent".

In this situation where there has developed secondary failure, 75% to 78% regain a durable response after dose intensification[73,98,99]. It is recognized that over time after a surgical resection "cure", most CD patients recur endoscopically, and fewer recur symptomatically. In the study by Regueiro *et al*[81], the endoscopic recurrence rate of 85% was reduced to 9% with continued IFX treatment.

TERTIARY FAILURES

If two TNFBs fail, there may be value in switching to a third TNFB[100], with 6 wk response seen in 58%. This, however, needs to be supported by further data[101]. In a European prospective cohort study of 67 adult CD patients who either lost response or developed intolerance to two TNFBs (usually IFX and ADA), treatment with a third TNFB resulted in a 6-wk response and remission rate of 61% and 22%, respectively[100]. "Concomitant therapy with corticosteroids or immunosuppressants order of anti-TNF administration, and prior history of response (i.e., primary non-response, secondary loss response or intolerance) to anti-TNFs did not affect patient's response to the third anti-TNF".

Table 19 Comparative efficacy of infliximab, adalimumab and certolizumab (%)

Induction	Response[1]	Remission[2]		Therapeutic gain
		TNFBs	PL	
Accent I	58	39	21	18
CHARM	58	46	17	29
Precise 2	64	48	29	19

[1]Weeks 2 to 6; [2]Weeks 26 to 30. Remission is often defined by the Crohn's disease activity index, but may also be defined using the Inflammatory Bowel Disease Questionnaire. PL: Placebo; TNFBs: Tumor necrosis factor-α blockers; CHARM: Crohn's trial of the fully human antibody adalimumab for remission maintenance.

The NNT for induction of remission was 9. In maintenance studies, when initial non-responders were excluded, NNT was 4 among responders, but NNT was 9 when including both responders and non-responders[3]. Peyrin-Biroulet *et al*[3] performed a meta-analysis of 14 placebo-controlled trials enrolling 3995 CD patients, of TNFB efficacy and safety in CD. The TNFBs included IFX, ADA, CER, ETA. Quoting directly from their report, "In overall analysis, anti-TNF therapy was effective for induction of remission at week 4 (mean difference, 11%; 95%CI: 6%-16%, $P < 0.001$) and maintenance of remission at weeks 20-30 in patients who responded to induction therapy and in patients randomized before induction (mean difference, 23%; 95%CI: 18%-28% and mean difference, 8%; 95%CI: 3%-12%, respectively; $P < 0.001$, for all comparisons)".

In adult CD patients who either lost response or became intolerant of two TNFBs given at optimal doses, when switched to a third TNFB, 22% achieved full remission and 51% continuing a clinical response to week 20[100].

How long to use TNFB

How long to treat with TNFB? Five observational studies have shown the response to IFX or ADA ranges from 73% to 100% at year one, 65% to 90% at year two, 61% to 68% at year three, and 52% to 58% at year five[102-105]. RCT data is not available yet for more than one year. Clear evidence based guidelines have not been developed as to when to stop TNF therapy, but reasonable empirical suggestions would be with loss of response or severe adverse effects to TNFB. Perhaps after one year TNFB treatment, when the patient is in clinical remission, there are no signs of active inflammation, and perhaps when mucosal healing is achieved, stopping TNFB therapy may be considered and discussed with the patient.

Comparative efficacy of IFX, ADA and CER

There are no head-to-head trials of one TNFB against another. There is heterogeneity in the CD patients recruited for the clinical studies, but interestingly the therapeutic gain for remission at weeks 26 to 30 were close to 18% to 23% (Table 19). It is unfortunate that these rates are so low.

Patient-reported outcome (PRO) instruments are used to provide information about health-related quality of life. The minimal clinically important difference (MCID) and minimal important difference (MID). PRO score changes have been determined from two TNFB clinical trials[106]. The MCID is the smallest difference in a PRO score that is associated with a clinically relevant difference or change[1,107]. The MID is the smallest difference in a PRO score that a patient can perceive as beneficial and would require a change in their treatment. The MCID and MID units are meaningful measures to assess alterations in PRO scores, rather than changes which albeit are statistically significant, are not necessarily useful in a practical sense.

When considering possible advantages of different TNFB, for example, ADA *vs* IFX, ADA has greater ease of administration (subcutaneous, self-administered *vs* infusion facility), no infusion reactions, higher remission rates among secondary non-responders to IFX[72,108], possibly reduced need for dose escalation over time [fewer secondary failures[109], higher later rates of response and remission (maintenance), lower cost [$ incremental cost-utility ratios (ICUR) per quality-adjusted life-year (QALY)][51], and lower risk of developing neutralizing antibodies. Again it must be stressed that there is no head-to-head direct data to allow for a definitive statement to be made about any comparison, including cost and patient preference. These conclusions are based on observations.

In contrast, the possible advantages of IFX *vs* ADA include no injection site reactions or infections, fewer numbers of individual adverse effects, higher early rates (induction) of response and remission, and high rate of fistula improvement or remission with both short- and long-term therapy. IFX also gave higher initial response and remission rates as compared to ADA. Comparatively, the use of ADA led to lower relapse rates, indicating that patients retained longer benefit from ADA *vs* IFX[51].

Continuation of immunosuppression with TNFB therapy

Most guidelines suggest that TNFB therapy be started when immunosuppression alone has failed. The used of TNFB plus immunosuppression is known as concomitant immunosuppression (CI). To understand the subtle aspects of the question about CI use with TNFBs in CD, we need to reflect upon several important studies.

Scheduled maintenance IFX treatment is more effective than episodic treatment (Accent I)[74]; Scheduled maintenance therapy is superior to episodic treatment in terms of the use of steroids or the use of hospitalization and surgery[44]. IFX induction plus azathioprine/6-mercaptopurine (6-MP) is more effective than azathioprine/6-MP alone in AZA-naive patients who are steroid-dependent (GETAID)[53]. Early treatment with IFX in combination with an antimetabolite is more effective than conventional therapy (step-up top-down)[110]. IFX plus azathioprine is significantly better for inducing steroid-free remission and mucosal healing than azathioprine alone in azathioprine-naive patients [study of biologic and immunomodulator naive patients in Crohn's disease (SONIC)][102]. In initial IFX responders, the maintenance of response falls from 83% at 30 wk, to 64% at 54 wk, and to 45% at 108 wk[111].

Table 20 Concurrent immunosuppression with azathioprine plus infliximab *vs* azathioprine alone in Crohn's disease[53] (%)

	Week		
	12	24	52
AZA	38	29	22
AZA + IFX x3	75[1]	57[1]	40
Therapeutic gain	37[1]	28[1]	18

[1]Significant difference. AZA: Azathioprine; IFX: Infliximab.

Scheduled maintenance IFX is more effective than episodic treatment[74]. The standard of care is to use TNFB on a regular and scheduled basis. The benefit of scheduled *vs* episodic use of IFX is related to there being lower levels of antibodies to IFX (ATI), fewer infusion reactions, and fewer hospitalizations and major surgery[54,112,113]. IFX induction plus AZA/6-MP is more effective than AZA/6-MP alone in AZA-naive patients who are steroid-dependent[53]. Early treatment with IFX in combination with an immunosuppressant is more effective than conventional therapy[110]. IFX plus AZA is significantly better for inducing steroid-free remission and mucosal healing, than is AZA alone in AZA-naive patients[114].

There is no additional benefit of adding MTX in the steroid-treated CD patient who is also on IFX[115]. It remains unknown if the continuation of MTX might be useful if the person were just on MTX + TNFB, rather than just being on GCS.

Most current guidelines recommend that AZA/MTX must have been used and be considered to have failed before TNFBs are introduced what should be. The duration of previous use of this immunosuppression and should AZA be continued TNFB once has been started is not known. Some data suggested that continuation of AZA after the introduction of IFX offers no additional benefit beyond 6 mo of combined therapy, as long as the TNFB is used on the basis of a regular schedule, rather than being given episodically. The authors of one trial suggested that "continuation of immunosuppression for more than 6 mo offers no clear benefit over scheduled IFX monotherapy"[116]. IFX monotherapy is superior to IFX plus immunosuppressive therapy after CD induction with IFX. In one study, four times more relapses occur with AZA maintenance after IFX induc-

tion, as compared with IFX induction and placebo maintenance[53]. In a second study, maintenance of remission of CD was no different when AZA plus IFX were given for maintenance, *vs* IFX alone[117].

In the study by Van Assche *et al*[116], at 6 mo in person on IFX in whom AZA had been withdrawn (IFX plus AZA D/C *vs* IFX + AZA), the CRP levels were higher, and IFX levels were significantly lower. Perhaps if the patients had been followed for longer than 6 mo, the AZA discontinuation group might have begun to experience more recurrences. The RAND appropriateness methodology, through a modified Delphi panel approach based on expert interpretation of the available literature, has been used with a "globally diverse panel of 13 gastroenterologists clinically experienced in inflammatory bowel disease"[118]. Using a total of 134 clinical scenarios, concomitant use of immunomodulatory was generally rated inappropriate for young males, and in some scenarios involving uncomplicated disease. However, CI were appropriate for those with extensive disease, shorter duration of disease, perianal involvement, prior surgery, females, and older patients (> 26 years)". In the GETAID study, the IFX + AZA (CI) gave a significant therapeutic gain of clinical remission off steroids, as compared with AZA alone, of 37% and 28% at weeks 12 and 24 respectively. But the benefit was lost at week 18 (Table 20).

Several important aspects to consider when attempting to answer the question of the benefit of using CI with IFX are the duration of follow up, whether the patient is naive to the immunosuppressants or the TNFB, whether the activity of the disease is high as suggested by an elevated surrogate marker such as CRP or mucosal lesions, what is using an endpoint of immunogeneity (such as antibody to TNFB or a disease activity index or QoL score), and whether the question is focused on the CD patient in whom you are trying to achieve induction or maintenance of remission. CI is clearly beneficial when added to IFX regimens which are episodic[112,119], but the controversy about CI comes from studies using regularly scheduled TNFB, the method which is new to the standard of care.

In the SONIC study, CD patients who were naive to both immunosuppression and to TNFB, the rate of clinical remission at week 26 was higher in those given IFX + AZA as compared with IFX alone or AZA alone (57%, 44%, and 31%, respectively)[120]. In a subgroup of patients who initially had both an elevated CRP and mucosal lesions at baseline, the IFX plus AZA + IFX alone values were even higher (69% and 57%, respectively). Since the difference between IFX + AZA was statistically greater than IFX alone, the data may be interpreted to indicate that CI is advisable, at least in the relatively short period of 26 wk, for steroid-free remission. However, the benefit of CI appears to fades after 6 mo to 12 mo[116,121].

In the infliximab maintenance immunosuppression discontinuation study (Van Assche), in CD patients who had been on AZA/MTX before starting IFX, stopping the immunosuppression at 6 mo did not have an adverse effect at 2 years to shorten the interval between IFX infusions, or the time to stopping IFX dosing. The prevalence of antibodies to IFX were similarly low in each group, and CI with IFX + AZA had no different effect in terms of mucosal healing.

The same message comes from the 2-year long COMMIT trial of steroid-requiring CD patients on IFX or IFX + MTX (Feagan 2010): CI does not provide a medium term (2-year) advantage. On the other hand, CI may increase the risk of adverse effects such as non-Hodgkin's lymphoma in adults[122], hepatosplenic lymphomas in children[123], as well as other concerns in children[124]. As well, CI did not benefit the medium term risk of surgery[125]. These individual studies suggest that in many CD patients, CI is "bridge" therapy for 6 mo while the full effect of TNFB is being achieved.

In an observational serial observation of 88 CD patients in remission and on IFX, half had their CI with AZA stopped. The IFX failure free status was 85% at 12 mo, and 41% at 24 mo and 32 mo[126]. This failure rate of IFX maintenance may have been due to the withdrawal of CI with AZA. Failure of CI on AZA-withdrawal was more likely to occur if the initial duration of IFX-AZA therapy was less than 27 mo, if the CRP was greater than 5 mg/L or the platelet count higher than 298 109/L. Until further data becomes available, we can be encouraged by "expert opinion" from the BRIDGE group analysis[118], suggesting that bridging CI may not be necessary such as in young males especially with limited disease, or some case scenarios including uncomplicated CD.

CI[127]treatment also reduces antibody to IFX (ATI) formation [24% *vs* 63%, on CI *vs* not on CI, $P = 0.007$; 43% *vs* 75%, $P < 0.01$]. Former researchers have proposed a time-structural treatment algorithm for moderate CD. This may of course need to be altered in future if "top down" (IFX→AZA→GCS) approaches to CD are shown to be beneficial.

The major role of CI may be in the patient who is on episodic IFX: in these persons, the incidence of ATI was 0% on CI *vs* 60%, not on CI, $P = 0.018$[128]. Comparatively, CI had no relationship on ATIs with scheduled IFX infusions. Combined immunomodulator with TNFB provides small therapeutic gain for 24 wk. Then, it is likely that CI can be abandoned without any detrimental effect of clinical response, and possibly a lower risk of adverse events. In fair balance for the issue of AZA discontinuation in the TNFB-treated patient, the "jury is still out", or as the Scots would say, the case is "not prove". Clearly, more data is needed.

In summary of Combination therapy: (1) In AZA and TNFB naive persons, with ↑ CRP or mucosal lesions on colonoscopy, remission and one year mucosal healing response to combination therapy (SONIC) A AZA = TNFB; (2) Unknown what to do after one year: for IFX alone after successful induction, maintenance for 54 wk gave a therapeutic gain of (28%-14%) 14% and 24% (38%-14%) for 5 mg/kg and 10 mg/kg IFX (Ac-

Table 21 Infliximab for fistulizing Crohn's disease: Induction and maintenance (%)

Study	Week of assessment	Outcome	IFX (mg/kg)		PL	TG
			5	10		
Present *et al*[75]	18	Response	68[1]	56[1]	26	42-30
		Closure	55[1]	38[1]	13	42-25
(ACCENT Ⅱ)	54	Response	46[1]		23	23
		Closure	36[1]		19	17

[1]Complete closure of draining fistulas; *P* value for each IFX group *vs* placebo group. For IFX 5 mg/kg, the TG for fistula closure ranged from 42% at 18 wk to 23% to 54 wk. Adapted from CADTH ('09). These differences are statistically significant. See Tables 3 and 4 of the CADTH report for *P*-values, number of participants and events. IFX: Infliximab; PL: Placebo; TG: Therapeutic gain.

Table 22 Adalimumab for fistulizing Crohn's disease induction, response and closure (%)

Study	Assessment week	ADA[1]	ADA[2]	ADA[3]	PL	TG
GAIN	Response					
	4	-	-	15	20	(-5)
	Closure					
	4	-	-	5	8	(-3)
Classic Ⅰ	Response					
	4	75	20	8	33	(42, -13, -25)
	Closure					
	4	75	0	0	17	(58, -17, -17)

[1]40 mg at week 0, 20 mg at week 2; [2]80 mg at week 0, 40 mg at week 2; [3]160 mg at week 0, 80 mg at week 2. ADA: Adalimumab; PL: Placebo; TG: Therapeutic gain.

cent Ⅰ). It is unknown what is best to do after one year treatment with IFX; (3) In post hoc analysis of combination therapy of immunomodulators plus IFX/ADA/certolizumab pegol (CZP) *vs* monotherapy with TNFB, there is no benefit in terms of induction synergy; (4) Because of study design, it is not possible to comment whether MTX plus TNFB is superior to TNFB alone; and (5) Not possible yet to predict which patient can be induced with TNFB and maintained on AZA (TNFB as a "bridge").

FISTULIZING DISEASE

Because between 20% to 40% of CD patients will develop fistulas[75,129], and because of the seriousness of perianal fistulas[78,129,130], the improved healing of fistulizing CD with TNFB would be anticipated to reduce the patient's lifetime need for major surgery. The presence of fistulas increases the risk of the need for colectomy[69,82]. Anti-TNF therapy is indicated for use in fistulizing CD. What is the evidence for IFX or ADA in this clinical setting? In the IFX infusion study by Present *et al*[75], IFX was given at 0 wk, 2 wk and 6 wk, and fistula response and complete closure of draining fistulas were assessed at 18 wk. As compared with placebo, IFX 5 mg/kg and 10 mg/kg gave 18-wk fistula response rates of 68% (P = 0.002) and 56% (P = 0.02) *vs* 26%, respectively. The corresponding closure rates were 55% (P = 0.001), 38% (P = 0.040), and 13% at 18 wk. For the 5 mg/kg dose of IFX for fistulising CD, this gave a therapeutic gain of 42% for both fistula response and closure.

Table 23 Adalimumab for fistulizing Crohn's disease maintenance (%)

Study	Assessment week	Combined ADA group[1]	PL	TG
CHARM	Closure			
	26	30	13	17
	56	33	13	20

[1]Statistically significant. Note that the two higher dose regimes of ADA do poorly for induction of fistulizing CD. The TG for IFX and ADA for fistula closure at 54-56 wk is a similar low value of 17%. PL: Placebo; TG: Therapeutic gain; ADA: Adalimumab; CHARM: Crohn's trial of the fully human antibody adalimumab for remission maintenance; IFX: Infliximab; CD: Crohn's disease. 140 mg q0w and 40 mg qw.

Table 24 Meta-analysis of efficacy and safety of biological therapies *vs* placebo (4 wk to 26 wk) in healing of fistulizing Crohn's disease

Treatment	Outcome (RR of fistulas remaining unhealed)
IFX, ADA, CER *vs* PL (4 wk to 26 wk)	RR = 0.88; 95%CI: 0.73-1.05; I^2 = 67%, P = 0.01 (considerable heterogeneity between studies, with no statistically significant difference in fistula healing at 4 wk to 26 wk, compared to placebo, when considering the 3 TNFB)
IFX *vs* PL (one trial)	RR = 0.62; 95%CI: 0.48-0.81
IFX, ADA, CER *vs* PL (excluding 2 trials reporting fistula healing up to only 4 wk)	RR = 0.80; 95%CI: 0.65-0.98; I^2 = 56%, P = 0.08 (heterogeneity between studies remained)
IFX (only 1 trial) *vs* > PL (followed until 54 wk)	RR = 0.81; 95%CI: 0.68-0.96

Adverse events could not be determined from data. CER: Certolizumab; IFX: Infliximab; ADA: Adalimumab; PL: Placebo; RR: Risk ratio; TNFB: TNF-α blockers; CI: Confidence interval.

In the IFX maintenance study by Sands *et al*[130] (Accent Ⅱ), IFX was infused every 8 wk until week 54. As compared with placebo, IFX 5 mg/kg gave 54 wk response rates of 46% *vs* 23% (P = 0.001); and the complete closure rates were 36% *vs* 19% (P = 0.009). This gave a 54 wk therapeutic gain of 23% and 17% for response and closure, respectively. Note the need to caution your CD patient with fistulizing CD: while early TG for response and closure was an encouraging 42%, at one year half this benefit was lost (falling to 23% and 17% at 54 wk)[75,78,129].

The TG in fistula response and closure for IFX (5 mg/kg) at 19 wk was 42% and 42%, respectively, with curiously lower rates of response for the 10 mg/kg IFX dose. At 54 wk, the TG for fistula response and closure was 23% and 17%, respectively. These are low values, but clearly IFX is of considerable benefit to individual patients who may have failed conventional therapy.

The 4-wk fistula response and closure rates for ADA (160 mg at week 0 and 80 mg at week 2) was not statistically different from placebo, but at weeks 26 and 56, the TGs were 17% and 20%, respectively. In the CHARM study, complete healing of fistulas was achieved in 30%

of those receiving ADA either weekly or every other week by week 26. This compared to 13% in the placebo group (P = 0.043). Recall that with fistulizing CD, the early response and closure with IFX was statistically significant, but was not so with ADA. At one year, the fistula response and closure rates were numerically similar for IFX and ADA, but the TGs were small for both TNFBs (Tables 21-23). Therefore, at this time, it is best to use IFX as 1st line to treat fistulising disease. There is insufficient data to recommend the same for CER. Meta-analyses have been performed for biological therapies in healing fistulising CD (Table 24). These authors suggested that "In preventing recrudescence of fistula, there was only one RCT reporting on this end point, which demonstrated that IFX was superior to placebo (Table 24). Although concerns about adverse events arising from the long-term use of these drugs are understandable, the data from the RCTs included in this meta-analysis did not demonstrate any increase in overall adverse events, or serious adverse events, with use for up to 1 year[61]."

In summary of the use of TNFB in fistulising CD "…. there is less evidence for the use of biological therapies, though there was a clear beneficial effect when only IFX was studied, although in one trial, and when only RCTs with follow-up in excess of 4 wk were considered in the analysis. There was also a benefit in favour of biological therapies over placebo when only studies that reported fistula healing in the longer term were included in the analysis."

THERAPEUTIC GAINS

Induction and maintenance, response and remission

At 2 wk and 4 wk after a single dose of IFX (5 mg/kg), the therapeutic gain (TG; IFX response minus the PL response) for clinical response was 56% and 64%, respectively (Table 7), and then fell to 36% at 12 wk[52]. The TG for remission was 36% and 46%, respectively, for weeks 2 and 4. When IFX (5 mg/kg) is given 2, 6 and every 8 wk until week 48[54] or 54[69,82], the week 54 TG for response ranged from 22%[54] to 30%[69,82], and for remission, the TG was 14% for IFX 5 mg/kg and 24% for 10 mg/kg. IFX doses of 10 mg/kg or 20 mg/kg did not give higher therapeutic gains in terms of response.

Thus, after excluding the approximately 30% initial IFX primary non-responders, about the two-thirds will respond and about one-third will go into remission at 4 wk (therapeutic gain). With continued 8-weekly IFX infusion for approximately one year, the TG for response is about one in four, and for remission one in eight. These represent relatively small gains which are not necessarily higher than reported historical figures for conventional therapy induction or maintenance therapy in CD. In children with CD, Kaplan-Meier analysis has shown that the cumulative probability of losing response to IFX after 1 year, 3 years and 5 years is 13%, 40% and 50%[131]. In adults with CD who chose to stop their IFX after achieving remission, 50% relapsed within 477 d after IFX discontinuation[132].

For the currently recommended dose of ADA of 160 mg at weeks 0 and 80 mg at week 2, TGs for response range from 15% to 19% at week 2, and 13% to 25% at week 4 (Sandborn *et al*[93]). The corresponding TGs for remission at 2 wk and 4 wk range from 10% and 14%-24%. The TGs for response at 56 wk range from 7%-25% when ADA is given as 40 mg q every other week, to 15%-33% when ADA is given as 40 mg q weekly[55,56,85,127,133]. The corresponding values for TG for remission at 56 wk range from a low of 4% to a high of 39%[55,56,85,127,133]. The values for the initial TGs in response and remission for induction therapy are numerically slightly lower for ADA; it is slightly higher for ADA for maintenance of response and remission. After two years of maintenance therapy with ADA every other week, 42% were in remission compared to 38% on placebo[134], and 50% with weekly ADA. This study also showed an improvement in IBD QoL questionnaires, suggesting clinical response. There is insufficient evidence at this time to recommend one type of ADA therapy over another.

In addition to therapeutic gains in terms of response and remission rates, other important clinical outcomes include improvements in quality of life, hospitalization and surgery. In patients with RA, combinations of TNFB with low-dose MTX are generally more efficacious than either drug used alone[135], the mechanism of anti-inflammatory properties of this combination is not totally clarified and it is not known if TNFB plus MTX have a synergistic mechanism[136], especially in CD.

Health utilization

Hospitalization: In Canada, about 20% of persons with CD have IBD-associated hospitalizations per year[2]. The proportion of patients who needed hospitalization for IBD were reported in three IFX trials and one ADA trial. Two IFX trials and one ADA trial reported the patients who needed surgery[51]. Overall, CD patients who received IFX maintenance therapy had an approximately 50% reduction in the rate and duration of hospitalization[74,129]. Lower hospitalization rates were also seen with ADA[93]. Infusing IFX on a scheduled *vs* an episodic basis reduces the rate of all-cause hospitalization, as well as IBD-related hospitalization. For example, in Accent I [74,137], the rate of IBD-related hospitalization was 28% in the episodic treatment group *vs* 23% in the IFX 5 mg/kg group (P = 0.047) and 24% in the 10 mg/kg group (P = 0.023). Furthermore, the number and duration of hospitalizations were lower in the scheduled *vs* episodic group (P = 0.018). In Accent II[129], the hospitalization rates were lower with IFX than placebo (14% *vs* 31%, $P < 0.01$), and 11% *vs* 33% in the initial IFX responders *vs* placebo ($P < 0.05$). Also, the mean duration of days in hospital was less with IFX *vs* placebo (0.5 d *vs* 2.5 d, $P < 0.05$).

In a population based study from the University of Manitoba of health care resource use among IFX us-

Table 25 Reduction (%) of health service utilization in Crohn's disease patients treated with Infliximab

	All CD patients[180]	CD fistula patients	
		(Rubenstein *et al*[180])	(Harrison *et al*[181])
ER visits	66	64	372
All surgery	38	66	
GI surgery	18	59	
Hospitalizations		59	37
Endoscopies	43		52
Radiographs	12	40	58-147
Outpatient visits	16	27	22-33

CD: Crohn's disease; ER: Emergency Room; GI: Gastrointestinal.

Table 26 Natural history of Crohn's disease and ulcerative colitis %

Natural history			
UC		CD[1]	
Remission at any point in time	50	Relapse	10-30
Overall intermittent course	90	Low activity	15-20
Remission: 3-7 yr after diagnosis	25	Remission	55-65
Yearly relapse	18	Chronic active	13-20
Intermittent relapse	51	Chronic intermittent	67-73
		Remission for 10 yr	13
10 yr colectomy rate	10	Surgery by 20 yr	> 75
Change in site of UC over 25 yr		Change in behavior	
LC→ C	50	NSNF → S	27
C → LC	75	NSNF → F	29

[1]Course 1 year after diagnosis. C: Entire colon; LC: Left colon; S: Structuring; F: Fistulising; NSNF: Non-stricturing, non-fistulizing; UC: Ulcerative colitis; CD: Crohn's disease.

ers, hospitalizations were higher in the IFX cohort until 18-24 mo after the first IFX prescription, at which point the rate of hospitalization in the IFX group fell to the level of the CD patients treated with AZA or GCS[138]. The likelihood of surgery was similar in IFX and GCS, which was higher than in AZA; even at 3 years post-closing. Overall physician visits were similar between IFX, GCS and AZA. Interestingly, ADA reduced hospitalization rates at 12 mo from 14% with placebo *vs* 10% and 3% with maintenance ADA given every other week and every week ($P = 0.12$ and $P < 0.01$, respectively)[115]. Hospitalizations account for more than 50% of the direct costs of CD[115]. Thus, in responders, TNFB reduced the rate of hospitalization, and this reduction in hospital days carries a major economic impact, which has to be countered against the high cost of maintenance TNFB requiring long-term use of drug, and the cost of management of adverse effects.

Surgery

CD-related surgery was more likely in the episodic *vs* the scheduled treatment groups (9% *vs* 3%, $P = 0.01$)[74], and the mean number of surgeries was higher (118 *vs* 60, $P < 0.01$). As compared with the placebo group, the mean number of surgeries in the IFX group was 13 *vs* 2 per

Table 27 Meta-analysis of efficacy and safety of biological therapies *vs* placebo in inducing remission in moderately to severely active ulcerative colitis[61]

Treatment	Outcome (RR of remission not being achieved)
IFX *vs* PL	RR = 0.72; 95%CI: 0.57-0.91 NNT = 4 (95%CI: 3-8)
Adverse event	
Any adverse event was no higher in IFX *vs* PL, and risk of serious adverse events was lower	RR of serious adverse event = 0.64; 95%CI: 0.41-1.00, $P = 0.05$ NNH = 13 (95%CI: 8-50)

IFX: Infliximab; PL: Placebo; RR: Risk ratio; CI: Confidence interval; UC: Ulcerative colitis; NNT: Needed to treat.

100 in all patients, and from 11 to 11 per 100 in the IFX initial responders[78]. With ADA maintenance, the 12-mo risk of major surgeries was 0.6% *vs* 3.8% in the placebo group ($P = 0.0005$)[115]. Data to date shows that TNFB reduce serious health utilization in persons with IBD.

TNFBS AND THE NATURAL HISTORY OF CD

It is not yet certain whether TNFB will achieve disease modification, or sustained mucosal healing[139]. CD is very heterogenic, and we need much more data on the possibility of patient risk-stratification based on environmental factors, disease phenotype, genotype and disease-initiating and-perpetuating factors (Table 25)[140].

A brief consideration of the use of TNFB in ulcerative colitis

There are many differences between CD and UC (Table 26). Only for purposes of comparison, a very brief overview is provided on IFX use in persons with UC, bearing in mind that these are separate and distinct conditions. About 20% of UC patients on 5-ASA maintenance therapy will have an acute attack each year, and about 10% of those with more severe symptoms will require hospitalization, usually for intravenous (IV) steroid therapy, cyclosporine or TNFB therapy; or surgery. About a third of these will not respond to IV steroids, and may be candidates for colectomy, cyclosporine or IFX.

In the Accent I and 2 studies, approximately half as many of those in the combined IFX group than placebo-treated UC patients required surgery (74% *vs* 8%), and the number of hospitalizations were also lower (18/102 patients *vs* 9/102 patients, $P < 0.01$)[74,115,129]. The colectomy rate in the placebo group is also approximately twice as high as in the IFX group: at 90 d, 67% *vs* 29% ($P = 0.017$)[141], and at 2 years, 76% *vs* 46% ($P < 0.05$)[142]. When IV GCS fail in patients with severe UC admitted to hospital for IFX[143], colectomy rates were as follows: in hospital colectomy, 24%; later colectomy, 14%; no colectomy, 62%. It is not clear how many will eventually require colectomy, whether they will need to

Table 28 Associations of mucosal healing in Crohn's disease

Fewer symptoms	
No previous surgery	
Previous surgery	
Less inflammation at 5 yr requiring steroid use	
Less use of steroids	
Steroid-free remission and no flare at years 3 and 4	Yes (TG, 44%)
Steroid-free remission, no flare and no TNFB at years 3 and 4	Yes (TG, 44%)
No new or persistently active fistulas	No

How do these associations with mucosal healing (MH) in CD compare with UC? About 90% of patients remain asymptomatic after achieving MH on once-a-day 5-aminosalicylic acid (Mezavant®), and after MH, 40% of UC patients remain asymptomatic (TG, 22%). TG: Therapeutic gain; CD: Crohn's disease; UC: Ulcerative colitis; TNFB: Tumor necrosis factor-α blockers (response in active treatment group minus response in placebo group; differences statistically significant).

Table 29 Rates of therapy-associated mucosal healing

Drug	CD		UC	
	Week[1]	MH, %	Week[1]	MH, %
5-ASA			4-8	25-71
Prednisone	4-9	0-27		
AZA	16-96	40-73	26	53-69
MTX	12-60	36-38		
IFX	4-104	29-100	8-52	62-71
ADA	12	27	8	30
CER	10	5-55		

[1]Time for endoscopic assessment of mucosal healing. CD: Crohn's disease; UC: Ulcerative colitis; MH: Mucosal healing; 5-ASA: 5-Aminosalicylic acid; AZA: Azathioprine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab; CER: Certolizumab.

be maintained long-term on IFX, or if they should simply be maintained on AZA or 5-ASA.

Primary non-response has been noted in 22% of UC patients treated with IFX[126]. IFX dose optimization was required in 45%, and colectomy was required in 19%. In a single-centre, prospective out-patient cohort of UC patients, response to induction therapy with IFX was 96% and 80% for ADA[144]. For maintenance therapy response to IFX and ADA, the rates were 78% and 70%. None of these differences was statistically significant.

Recent meta-analysis has considered the efficacy and safety of biological therapies in inducing remission in moderately to severely active UC (Table 27)[61].

The summary points are as follows: (1) No data on the efficacy and safety of biological therapies *vs* placebo in preventing relapse of disease activity in quiescent UC; (2) "IFX was highly effective in inducing remission in patients with moderate to severely active UC who had failed therapy with first and second-line agents, and who had also failed to respond to a course of high-dose corticosteroids, with a NNT of only 4[61]"; (3) IFX for rescue therapy in severe UC, with a therapeutic gain of (71%-33%) 38% (NNT, 3); (4) IFX will induce and maintain clinical remission and mucosal healing in treatment–refractory, moderate/severe UC; (5)Unknown if

Table 30 Complete mucosal healing[1]

	ADA	PL [ADA induction only (3 doses)]	TG (%)
ITT		PL	
week 12	27%	13% (P = 0.056)	14
week 52	24%	0% (P < 0.001)	24
PP			
week 12	28%	13% (P = 0.046)	15

[1]Extend: Open-label adalimumab[182]. Of patients with CMH at week 12, 46% of ADA every other weeks had CMH at week 52 *vs* 0% CMH in those receiving only ADA induction. ITT: Intention-to-treat; PP: Per protocol; TG: Therapeutic gain; PL: Placebo; ADA: Adalimumab.

IFX plus AZA is superior to monotherapy with AZA or IFX; (6) Before starting IFX in UC, discuss options of AZA, cyclosporine A (Cy A), and pan proctocolectomy with ileoanal pouch procedure; (7) Unknown comparison of Cy A *vs* TNFB for rescue therapy; (8) Short term: young age, absent pANCA (in CD, presence of pANCA, ASCA, or pANCA + ASCA had no predictive valve); and (9) Long-term (predictors for no colectomy): short term clinical response to CRP < 5 mg/L, no previous treatment with IV steroids or Cy A.

Importance of mucosal healing

There are a number of potential advantages in achieving mucosal healing in CD (Table 28). In both CD and UC, the rates of mucosal healing range widely depending upon the therapeutic agent and the duration of treatment (Table 29).

In clinical trials, there has been no uniform standard for defining mucosal healing (MH), no agreed-upon optimal time for assessing MH. It is not surprising then that the ranges of MH are very wide.

The clinical trials of agents used to treat IBD have used a variety of clinical scores heavily based on symptoms (CDAI), measures of QoL, appearance of the mucosa on diagnostic imaging, endoscopic appearance, or histopathology. Some patients with CD treated with TNFB achieved MH and the question was raised whether MH by itself, independent of patient's symptoms or QoL, was an important outcome variable. MH would be an important outcome if it were to be shown that it was associated with reduced risk of recurrence (i.e., greater likelihood of maintenance of remission), reduced risk of extraintestinal complications, reduced risk of medical treatment-associated complications, long-term risk of hospitalization, surgery or development of colorectal cancer. The risk of development of stricture or fistula in CD increases over time[46,145], so maintaining mucosal healing may decrease these problems. However, there is no evidence to answer the above asked questions.

It seems self-evident that MH should become an outcome objective of therapy in IBD. However, this needs to be proven. Because the prevalence of MH may decline over time, and the number of the week of study repeating MH varied widely, it is not possible to assert which class of drug has the highest rate of MH (Table 29), nor is it plau-

Table 31 Meta-analysis efficacy of 5-aminosalicylic acid/sulfasalazine to induce remission or to prevent relapse in Crohn's disease

	Induce remission (RR of not being in remission) on active therapy	Prevent relapse (RR of relapse)
CD	SASP (only 2 RCTs) 0.83 (95%CI: 0.69-1.00; trend) 5-ASA 0.91 (95%CI: 0.77-1.06; not significant)	SASP/5-ASA, ITT analysis, no benefit 5-ASA, PP analysis: RR = 0.79 (95%CI: 0.66-0.95; NNT = 13 to prevent relapse)
UC	0.79 (95%CI: 0.73-0.85; NNT = 6)	0.65 (95%CI: 0.55-0.76; NNT = 4 to prevent relapse)

CD: Crohn's disease; ITT: Intention-to-treat; PP: Per protocol; SASP: Sulfasalazine; 5-ASA: 5-Aminosalicylic acid; CI: Confidence interval; UC: Ulcerative colitis; NNT: Needed to treat; RR: Relative risk; RCT: Randomized controlled trial.

sible to compare IFX with ADA or CER. In an open-label study of ADA in which there was a "placebo" (3 doses of ADA for induction), the therapeutic gain in the 12 wk complete mucosal healing (CMH) was 14% in the intention-to-treat analysis (ITT) and 15% in the per-protocol analysis, with 52 wk ITT CMH of 24% (Table 30).

When and how to start TNFB therapy: Start early, vs follow the current guidelines

This question may also be considered as "step-up" *vs* "start high". When considering a comparison in outcomes from a management approach starting early with biological therapy in the patients' lifelong CD course, *vs* the standard of care of step-up therapy, we must reflect the strength of the evidence supporting each of the components of the traditional approach in CD of using 5-ASA products, antibodies, GCS, and the immune suppressants AZA and MTX. The excellent systematic reviews and meta-analyses in the "Red Journal"[61] are very helpful in this regard, and will be reviewed briefly here to demonstrate the potential limitations of the currently accepted standard of care.

5-aminosalicylates therapy: In contrast to the supportive data in UC for the use of 5-ASA to induce remission and to prevent relapse, the data for CD is poor (Table 31)[61]. Thus, for the usually accepted ITT analyses, neither SASP nor 5-ASA can any longer be recommended in persons with CD to induce or maintain remission.

Antibiotic therapy: There is diversity in the design of antibiotic studies in IBD, including which antibiotic, as well as dose and duration of therapy. The antibiotics often used in clinical practice for this indication were metronidazole, cipro', and rifaximin (not yet available in Canada). While taking into account these events, it may surprise some readers that antibiotics are superior to placebo for the induction and maintenance of remission in CD, as well as induction of remission UC (Table 32)[146].

Glucocorticosteroid therapy: Standard GCS remains

Table 32 Efficacy of various antibiotics to induce remission or to prevent relapse in Crohn's disease

		Induce remission (RR of not remission) on active therapy	Prevent relapse (RR of relapse)
CD	Inflammatory disease	0.85 (95%CI: 0.73-0.99, P = 0.03)	0.62 (95%CI: 0.46-0.84)
	Perianal fistula	0.80 (95%CI: 0.66-0.98)	
UC		0.64 (95%CI: 0.43-0.96)	

UC: Ulcerative colitis; CD: Crohn's disease; CI: Confidence interval; RR: Risk ratio.

the standard for inducing remission in moderate-to-severe UC, but must never be used for maintenance of disease because of its lack of efficacy and its high adverse effects risk profile. While the trials of GCS in active CD individually showed efficacy because of heterogeneity between studies (I^2 = 88%, P = 0.004) and the small size of the studies. When the risk difference was used as the summary statistic, the treatment effect became significant, with NNT = 3 (95%CI: 2-11)[61]. Furthermore, systematic review and meta-analysis showed that standard GCS were superior to the locally acting budesonide in achieving remission in active CD (RR = 0.82; 95%CI: 0.68-0.98); oral budesonide itself was superior to placebo for CD remission (RR = 0.73; 95%CI: 0.63-0.84). Oral budesonide used for up to one year delays the time to recurrence of active CD.

Adverse effects of GCS therapy in the setting of treating active CD could not be determined in the studies of Ghosh *et al*[34] and Summers *et al* Standard GCS-related adverse effects were more frequent than with budesonide (RR = 1.64; 95%CI: 1.34-2.00). Thus, GCS and budesonide will continue to be used for short term treatment of active CD, and the choice between standard GCS *vs* budesonide must be made on the trade-off of efficacy, adverse effects and cost.

Immunosuppressive therapy: The meta-analysis of the use of Immunosuppressants in CD was disappointing; example (Table 33), the relative risk of not being in remission for active CD was not different from placebo. However, if OR was used rather than RRs as the summary statistic, then the result was significant, using a random effects model (OR = 0.42; 95%CI: 0.2-0.89). While some clinicians would accept significant ORs of AZA/6-MP/MTX *vs* placebo as reason to use immunosuppression to induce remission of active disease, the onset of biological benefit takes 2-3 mo. While many gastroenterologists use AZA/6-MP/MTX to prevent relapse in CD, this is likely based on the evidence of individual trials or previous meta-analysis. Surprising to some, while immunosuppressant therapy in UC was not significantly superior to placebo to induce remission, azathioprine was efficacious to prevent relapse (Table 33).

In summary then of the standard step-up approach to the care of patients with CD, in the treatment of active disease to achieve remission, the step using 5-ASA/

Table 33 Meta-analysis of efficacy of immunosuppressive therapy to induce remission or to prevent relapse in Crohn's disease and ulcerative colitis

		Induce remission (RR of not being in remission on active therapy)	Prevent relapse (RR of relapse)
CD	AZA/6-MP *vs* placebo/no therapy	0.87 (95%CI: 0.71-1.06)	0.64 (95%CI: 0.34-1.23)
		If OR was used rather than RRs as the summary statistic, then "the result was significant, using a random effects model (OR = 0.42; 95%CI: 0.2-0.89)	There was no statistically significant benefit for continuing AZA to prevent CD relapse (RR = 0.39; 95%CI: 0.21-0.74)
	MTX *vs* placebo	0.82 (95%CI: 0.65-1.03), no statistically significant benefit	IM MTX: 0.57 (95%CI: 0.35-0.94); NNT = 4 (95%CI: 2-25) to prevent one relapse
		If or used, then the result was statistically significant: OR = 0.47; 95%CI: 0.23-0.99	PO MTX to facilitate steroid withdrawal: 0.82 (95%CI: 0.58-1.17)
	Cyclosporine *vs* placebo (only one trial)	0.84 (95%CI: 0.62-1.07; tacrolimus, one study, no improvement: RR = 0.64; 95%CI: 0.44-0.92)	0.96 (95%CI: 0.77-1.20)
UC	AZA *vs* placebo	0.85 (95%CI: 0.71-0.01, *P* = 0.07)	0.60 (95%CI: 0.37-0.95, *P* = 0.03); NNT = 4, 95%CI: 2-10)
	MTX *vs* placebo	0.59 (95%CI: 0.04-7.90)	

UC: Ulcerative colitis; AZA: Azathioprine; MTX: Methotrexate; RR: Risk ratio; OR: Odds ratio; NNT: Needed to treat; CI: Confidence interval; IM: Intramuscular; PO: Oral; CD: Crohn's disease; 6-MP: 6-mercaptopurine.

Table 34 Benefits of mucosal healing in Crohn's disease[183]

Positive: IBSEN, Step-up/top-down, SONIC
IBSEN (Froslie): 5-yr longitudinal study of IBD patients not on TNFB and enjoying mucosal healing
CD: ↓ Inflammation, ↓ Future use of steroids
UC: ↓ Risk of future colectomy
Negative: Music

IBSEN: The inflammatory bowel south-eastern Norway (study group of gastroenterologists); SONIC: Study of biologic and immunomodulator naive patients in Crohn's disease; TNFB: Tumor necrosis factor-α blockers; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

Table 35 SONIC (Crohn's disease diagnosed within 4 years) %

Conventional step-up (steroids→AZA→IFX)	Step-down (IFX x3, with AZA)		
2 yr	30	73	(43)
3-4 yr			
No flare, no steroids, on demand IFX	about 65	about 20	(about 45)
No flare, no steroids, no TNFB	about 60	about 15	(about 45)
New or persistent fistulas	about 2	about 22	(about 20)

IFX: Infliximab; AZA: Azathioprine; TNFB: Tumor necrosis factor-α blockers.

SASP should be removed; the antibiotic and GCS/budesonide steps remain, and the AZA/6-MP/MTX step is rather shaky and quite slow.

In the end, we may believe that early use of biologics is too aggressive a step, which needs to be shored up by more data, but we need to acknowledge that the standard step-up approach is not as good as we used to accept.

The presence of two mutant nucleotide-binding oligomerization domain-containing protein 2 (NOD2) alleles may be quite specific for future complicated disease. Although the sensitivity is poor and the area under the sROC curve is poor, the high degree of specificity may be sufficient to recommend testing for double mutations if we can identify therapies that can truly change the outcomes for this high-risk stratum of patients. Testing for double mutations would likely miss many patients with aggressive disease. However, the presence of double mutations, particularly mutations in P.Leu1007fsX1008, does identify a high risk stratum of reagent-grade patients that should be identified and analyzed as a distinct subgroup in clinical trials. If prospective trials can show a change in outcomes in these patients, this would be a strong justification for targeted top-down therapy in this group of high-risk patients to prevent stricturing or fistulising complications.

The other major finding of this meta-analysis is the lack of prognostic value of the presence of one mutant allele for predicting CD behaviour. Although we specifically studied NOD2 mutations, recent genome-wide association studies have identified numerous other genes that contribute to the susceptibility of developing CD. It is likely that the level at which genetics contributes to phenotypic disease expression is complex and multiple risk alleles contribute to complex interactions. Further studies addressing disease behaviour will likely require large-scale, multicenter trials to obtain sufficient clinical data to accurately ascertain the cumulative risk from clinical, genetic, and environmental factors. Once additional predictive factors are identified and validated, we will be better able to study targeted therapies in high-risk patients.

In a prospective extension of a patient cohort study of 133 newly-diagnosed CD patients recruited from 18 European Centres, half of the patients were initially treated with as "top-down", and the other half as step up[147]. In the 46 persons who had endoscopically proven mucosal healing at two years, ileocolonoscopies were continued at year three and four. The results can be interpreted as being either for or against mucosal healing as an important therapeutic outcome: complete mucosal healing at year two was associated with a greater proportion of steroid-free clinical remissions (71% *vs* 27%, respectively; *P* = 0.036). While MH may be associated with

Table 36 Sonic naive (no azathioprine, no tumor necrosis factor-α blockers) %

	Clinical remission, off steroids		
	AZA	IFX	AZA + IFX
26 wk	30	44	57
50 wk	24	35	46
	17	30	44[1]

[1]AZA + IFX *vs* IFX (P = 0.55) or *vs* AZA ($P \leq 0.001$). IFX: Infliximab; AZA: Azathioprine.

later steroid-free remission, it is not clear if this correlates with fewer adverse effects, or better outcomes such as QoL, hospitalizations or surgery. There are studies in which the support for the benefit of MH is positive, or negative (Table 34). The case remains "unproven" about the long-term importance of mucosal healing rather than symptom relief as the major end point of treatment of CD.

In accordance with recommendations from current guidelines, the TNFBs are usually initiated in the CD patient who has failed the "step up" approach of sequential use of GCS, ASA/MTX. Initial clinical trials of TNFB were designed to take this conventional sequence into account, and to use TNFB as "add-on" therapy. The terms "step-up, step-down" "top-down" and "fast forward" all related to the concept of whether CD patients will do better in the long-term if using TNFBs earlier *vs* relatively later after the diagnosis of CD, in an attempt to possibly alter the course of the disease and to thereby improve patient outcomes.

Is the response to TNFBs greater when used early in the history of the person's CD? Is there benefit of starting recently diagnosed CD patients on IFX or IFX+AZA immediately, i.e., "top down" *vs* the "step-up" of GCS→AZA/MTX→TNFB? In the open label trial of D'Haens *et al*[110], newly diagnosed CD patients were treated either with AZA + IFX, or conventional therapy with GCS, followed by AZA, and then followed by IFX (GCS→AZA→IFX). With the early use of AZA + IFX with GCS (top-down), at week 26 and 52 the proportion who were steroid-free and who had not undergone IBD-related surgery was 60% and 62%, respectively. In the step up group, the corresponding figures were lower at 36% (P = 0.006) and 42%, respectively. Although the top down group achieved remission faster than did the GCS-AZA-IFX group (P = 0.018), at 53 wk a similar percent of patients in each group were in remission (Table 35).

What then is the influence of confounders such as CD duration, or being TNFB- or AZA/MTX naives? In a post-hoc analysis of the use of ADA in the Charm study[57], after adjustment for potential confounders, disease duration had an effect on clinical remission (RR: 0.96, 95%CI: 0.94, P = 0.002). In the Sonic study (REMICADE), patients with CD who had not previously received treatment with anti-TNFBs or immunomodulators (anti-TNF naive as well as anti-AZA/MTX naive) were randomized to IFX, AZA, or IFX + AZA. At 26 wk, the steroid-free remission rate was higher with IFX monotherapy (P = 0.022) or AZA monotherapy (P = 0.009) groups (Table 36).

When IFX was used within one year of the diagnosis of CD, 78% of the early treatment group responded and 76% were in remission, as compared to 47% and 37% (P < 0.001 and P = 0.002, respectively)[51]. This suggests that earlier use of TNFB may be better when looked from the perspective of this relatively short term follow-up. But of course, those persons started earlier on TNFBs might possibly have more total adverse events (AEs), because of their potentially longer duration of total exposure to drug.

Clearly, current guidelines must be respected. More data is necessary, but it looks promising that the early use of TNFBs (or AZA) may provide outcome advantages. If this possibility becomes evidence based, then guidelines and clinical practice will need to change appropriately.

ADVERSE EFFECTS

Anti-IFX antibodies

In some CD patients who develop secondary non-response to TNFB, this may be due to the development of neutralizing antibodies, what proportion of patients on IFX or ADA develop antibodies to IFX (ATIs), or antibodies to ADA [antibodies to adalimumab (ATAs)]? In patients given scheduled IFX 5 mg/kg, 10%-16% develop ATI, whereas ATA occur in 0.04%-2.60%. Infusion reactions with IFX are more common if there are ATI (36%-50% with, and 21%-24% without ATI). Concurrent immunosuppressant (CI) therapy reduces ATI (63%-75% with concurrent AZA/MTX, *vs* 24%-43% with no concurrent AZA/MTX). This beneficial effect of concurrent immunosuppressant on the rate of development of ATI or ATA is most pronounced with episodic rather than with the scheduled use of IFN. This is because the development of ATIs is greater with episodic than with scheduled infusion of 5 mg/kg or 10 mg/kg IFX: 30%, 10%, and 7%, respectively (P < 0.001)[113]; 39% episodic *vs* 16% scheduled (P = 0.036)[128].

The presence of ATI/ATA is of clinical importance. In fact, there is a negative correlation between the concentration of ATIs and the duration of response to IFX (P < 0.001)[112]. Furthermore, the median ATI concentration was 15-fold higher in those with loss of response to IFX than in those who maintained response (P < 0.0001)[148].

The development of ATAs is uncommon: in Classic Ⅰ, the rate of ATA formation at week 4 was 0.04%[149]. In Classic Ⅱ, 2.6% showed ATA[55]. The presence of ATA is also clinically important: there is a significant relationship between the presence of ATAs and non-response to ADA (OR = 12.7, 95%CI: 1.7-92.6, P = 0.05)[83].

Protection against the development of ATIs may be achieved with intravenous hydrocortisone premedication given immediately before each IFX infusion, with lower

Table 37 Adverse effects for infliximab and adalimumab[1]

	IFX	ADA
All AEs	-	-
Serious AEs	-6.6	-4.7[2]
Nausea	+7.9[3]	+2.4
Pharyngitis	+16.1	+3.7[2]
Injection site infection	-	+3.8[2]
Injection site reaction	-	+10.5[2]
Arthralgias	-	+4.3[2]
Urinary tract infections	-	+3.5[2]

[1]The rates are shown as the percentage differences in the rates of AEs in tumor necrosis factor-α blocker minus placebo groups. AEs are shown, with the increased (+) or decreased (-) difference from placebo. Note that the duration of follow-up observation varied between study, and has not been standardized here (e.g., per 100 treatment years); [2]The statistically significant or trends: (1) Note that the rates of all AEs as well as serious AEs were lower than in the placebo groups; and (2) Serious AEs include lymphoma and other cancers; [3]Significant difference. IFX: Infliximab; ADA: Adalimumab; AEs: Adverse effects.

median levels of ATI[150]. However, in this study, even though ATIs were lower, the use of hydrocortisone did not affect the rate of infusion reactions or increase clinical response. As noted previously, the major role of concurrent immunosuppression with maintenance TNFB may be when TNFB is not given in the recommended scheduled manner. Therefore, due to higher rate of ATI with episodic treatment, it is not a recommended route of therapy.

Other adverse effects

When considering the safety of TNFB, it is important to consider whether the TNFB was given with immunosuppressants or steroids, and for how long therapy was undertaken. In one study, steroid use in persons on IFX were the only independent risk factor for infections [or 2.69 (95%CI: 1.18-6.12, $P = 0.018$)][151]. The overall rate of AEs in IFX-treated patients is about 13%[77,111,151,152], which is not statistically significantly different from the 19% AE rate quoted for non-IFX treated patients [OR = 1.33 (95%CI: 0.56-3.00, $P = 0.45$)][151].

In a case-controlled family practice, population-based retrospective study of 15 471 IBD patients followed for an average of 6.4 years, AZA did not increase the risk for solid organ cancer development (OR = 1.04, 95%CI: 0.89-1.21), but the risk of lymphoma was enhanced 3.22-fold[153]. If myelosuppression occurs with AZA in naive CD patients, it does so in the first 6 wk of AZA therapy[154].

AE data has been collected from 10 TNFB randomized controlled trials in IBD [(five IFX[45,52-54,69], four ADA[55,56,85,93] and one with etanercept[31]). More AEs (serious AEs, pharyngitis, urinary tract infection, injection site reaction or infection)] were reported with ADA *vs* placebo than IFX *vs* placebo (Table 37). From four ADA placebo-controlled trials[54-56,93], there was no difference between ADA and placebo in the percentage of persons with overall AEs (77% *vs* 80%, $P = 0.145$). AEs that were more common in the ADA than the placebo group were pharyngitis (10% *vs* 6%, $P = 0.047$), injection site infection (4.9% *vs* 1.1%, $P = 0.005$), injection site reaction (22% *vs* 12%, $P = 0.003$), arthralgia (10.4% *vs* 6.1%, $P = 0.014$) and urinary tract infection (5.0% *vs* 1.5%, $P = 0.017$) (Table 37). Curiously, ADA had significantly fewer serious AEs than did placebo (6% *vs* 10%, $P < 0.001$), and the placebo group from one study had numerically more AEs than another placebo group (11% *vs* 18%, $P = 0.065$).

The medium-term safety of TNFB has been reviewed: In 21 studies enrolling 5356 CD patients, TNFBs did not increase the risk of serious infection malignancy or death[3]. However, as cautioned by these authors, "definitive conclusions cannot be drawn because of methodological limitations (safety analyses satisfied by concomitant therapy were not performed in the individual studies". Fortunately, not all trials reported death as an outcome. In ACCENT I [54], with IFX 5 mg/kg, two patients unfortunately died from sepsis and one from a myocardial infection.

The most common AEs with CER were upper respiratory tract infection (20%), urinary tract infection (7%) and arthralgia (6%)[155]. From five IFX placebo-controlled trials[52-54,78,156], there was no difference between IFX and placebo in the percentage of persons with overall AEs (93% *vs* 95%, $P = 0.355$). The absolute serious AE was 7% higher in IFX *vs* the placebo groups (24% *vs* 17%, $P = 0.019$), and only sinusitis was reported more frequently in UC given IFX *vs* placebo (9% *vs* 5%, $P = 0.04$). Concomitant immunosuppression with maintenance IFX does not significantly extend the duration of clinical response[111]. Nausea was the only AE which was more frequent in IFX than placebo (15% *vs* 7%, $P < 0.031$), with a higher trend for pharyngitis (18.9% *vs* 2.8%, $P = 0.056$). Importantly, the increased role of infections in CD or in CD patients on IFX may be from their concomitant use of GCS[129,151].

Life-threatening side effects occur in 2%-5% of persons on IFX, with twice the rate of sepsis, myelotoxicity or risk of lymphoma as those on thiopurines[157]. However, IFX has been used by approximately one million persons worldwide. As a class, the main complications of TNFBs are infections, lymphoproliferative disorders and malignancy[158]. As well, neurological, dermatological, autoimmune and cardiac complications commonly occur with these agents (Table 37). Deaths have been reported in persons using TNFB. The risk of sepsis, malignancies and myelosuppression is higher if AZA/MTX is given with TNFB. For example[159], the risk of opportunistic infections is increased with TNFB (OR = 4.4), and is increased much more if two or more immunosuppressants and used (OR = 12.9).

It is recommended that the complete blood count (CBC) be performed at 1 wk, 2 wk, 3 wk, 4 wk, 6 wk and 8 wk after starting AZA therapy, and then to continue a monthly CBC thereafter. During the first 8 wk of AZA therapy, the risk of leucopenia (white blood cell, $< 1.0 \times 10^9$/L), neutropenia (platelet-neutrophil complexes, $< 1.0 \times 10^9$/L), and thrombocytopenia (platelet count, $< 20 \times 10^9$/L) were 0.3%, 1.0% and 0.2%, respectively[154]. For

treatment intervals of greater than 26 wk, these hematological risks were 0.2%, 1.1% and 0.1%.

IBD patients, including those on GCS and AZA/MTX, have an increased risk of TB (RR = 2.36, 95%CI: 1.17-4.74). TB reactivation is an accepted risk of TNFBs, with the RR for reactivation 2-20 times that of the general population[160,161]. In high-risk areas, IBD patients who are scheduled to be placed on TNFBs must be screened for histoplasmosis and coccidiomycosis[162].

Some authors suggest that CD itself, in the absence of the use of AZA/MTX/TNFB, does not have an increased risk for the development of lymphoma[163]. Others would argue that CD itself does carry a small increased risk. However, these drugs used to treat CD certainly are associated with as much as a 4-fold increased risk of the development of lymphoma[157]. And yet, in the Treat registry of IBD patients, the incidence of malignancies was 0.53 per 100 patient years in the IFX group and 0.49 in the non-IFX group[164]. A small number of adolescents with CD on IFX and thiopurines have been reported as developing hepatosplenic T-cell lymphoma[165]. This consensus is that the concurrent use of both AZA/MTX plus TNFB should not be used in non-adults.

ECONOMIC EVALUATION

Within the context of an economic evaluation, there are several special considerations: (1) Safety (immunizations complete, no TB, no personal/ or family history or demyelation disorders; (2) Perhaps early after the diagnosis of IBD has been made; (3) Perhaps in those with predictors of likely response to TNFB; (4) About 1/3 of persons treated with IFX, ADA and CZP will have a primary failure; (5) Use same TNFB in higher dose or same dose more frequently or, switch to another TNFB; (6) With a secondary failure, the initial response to another TNFB is lower; and (7) Exclude concurrent infection/abscess as a reason for "failed" effect on luminal inflammation.

Anti-TNF therapy is expensive. While there are reductions in all areas of disease-related resource use following IFX therapy, in the United Kingdom, this was not sufficient to cover the cost of TNFB therapy[166]. Future studies of these pharmacoeconomic issues will also need to include considerations on indirect cost-savings and changes in the quality of life[167]. A cost-utility analysis was determines as a part of the CADTH report, giving cost per QALY. The cost of usual standard of care (SOC) was $17 107, that for ADA was $45 480, and for IFX $54 084. The QALY for SOC was 2.555, and the value for ADA and IFX were only slightly higher at 2.701 and 2.721, respectively. This resulted in ICUR of $193 305 and $222 955 for ADA and IFX, respectively. When comparing IFX with ADA for the ICUR efficiency using a five-year time horizon, the values were $193 305 *vs* $451 165. These cost efficiencies of IFX *vs* ADA were not so much due to variations in the values of their QALY, but rather to higher initial remission and response rates with IFX including with fistulizing CD, lower relapse rates with ADA, and higher remission rates amongst the patients who stopped responding to initial IFX and were switched to ADA[72,108]. It is important to stress that this comment is based on very preliminary data. From an economic perspective, it is preferential to switch an IFX non-responder to ADA, rather to increase the dose from 5 mg/kg to 10 mg/kg. There is insufficient evidence of the use of treating an IFX non-responder to a reduced dose interval. Also, the lower rates of hospitalization and need for IBD-related surgery in the TNFB groups was partly offset by the higher drug costs. The true economic impacts of SOC *vs* TNFBs will depend upon longer term data.

For interest, the cost of usual care for UC patients was $24 268 and the QALY was 2.015. For a strategy of IFX 5 mg/kg every 8 wk for maintenance, and for those who do not respond to IFX or lose the initial benefit, the patient is switched to ADA. This escalated the cost to $82 756 and is associated with a very small increase in QALY from 2.015 to 2.178. For an incremental QALY of 0.163, the ICUR was an astounding $308 088. In contrast, an economic evaluation in the United Kingdom gave a more favourable ICUR for IFX of about $70 000 (using the 2008 currency exchange rate). While there are other economic evaluations of TNFB[168-170], the CADTH study is the first to compare the initiation and maintenance therapy costs of IFX and ADA. The authors of the CADTH review suggested, with regards to the economics of the use of TNFBs, that "...the incremental cost-effectiveness in managing this IBD patient population may not be perceived as favourable for anti-TNFα drugs compared with usual care without these drugs. This finding was supported by deterministic sensitivity analysis and probabilistic sensitivity analysis".

Over the past four years (2006 to 2010), and based on acceptable incidence and prevalence estimates of CD and UC[2], the number of IBD patients has risen by about 5% (1% per year), yet between 2006 to 2008, the annualized increases in public spending on IFX and ADA in IBD have risen 25% and 18%, respectively. There is no evidence that over the past five years there has been a marked increase in the number of IBD sufferers who have suddenly developed severe or non-responsive disease. This 5-fold greater use in TNFBs as compared with the increase in the number of sufferers may reflect the wider dissemination and acceptance of TNFB guidelines, or may be the result of inappropriate over usage.

The economic analysis of the health services impact suggest that "…Adalimumab and IFX for the treatment of IBD may not be perceived to be a cost-effective use of health care resources"[51]. There are no head-to-head trials comparing standard-of-care *vs* anti-TNF therapy. What do TNFBs add to the annual cost of care of every IBD patient in Canada? "Based on trends in expenditures over the past three years, total public expenditures on anti-TNFs for IBD are projected to be $94 832 652 in fiscal year 2010-2011. The estimated annual drug costs for IBD patients who are on maintenance therapy with

Table 38 Some of the important anti-tumor necrosis factor studies still to be reported

Direct head-to-head comparisons of various anti-TNFs
Further evidence that treating with anti-TNFs earlier after the diagnosis of CD is better
Further clarification of the role of using scheduled anti-TNF therapy without concomitant AZA/MTX
Head-to-head trials comparing the effectiveness of IFX, ADA, and CER peg with conventional therapy in refractory CD (other than newly diagnosed and treatment naive patients)
Long-term safety
Evidence for efficacy of switch from ADA → IFX for ADA secondary loss of response
Optimal therapy for anti-TNF therapy primary non-responders
The comparison of standard-of-care *vs* anti-TNF therapy for maintenance therapy in UC
Are physicians correctly distinguishing mild from moderate or severe CD?
What is the profile of the patient who is most likely to respond? Are they genotypic or phenotypic characteristics?
When is the optimal time in the course of the patient's illness to begin anti-TNF therapy?

TNF: Tumor necrosis factor; CD: Crohn's disease; AZA: Azathioprine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab; CER: Certolizumab; UC: Ulcerative colitis.

anti-TNF drugs vary from $23 000 to $38 000[159]. Similar benefits of IFX on rate of hospitalization, surgery and procedures were described in the Accent I and II trails[54,164].

This reduction in health serious utilization could be partially offset by the high cost of biologics, so that cost effectiveness and cost-utility analysis are necessary. A UK cost utility analysis reported that maintenance treatment with IFX every 8 wk was cost-effective for patients with luminal and fistula CD[171]. For example, for luminal CD, an incremental cost per QALY gained for IFX was £26 128 (using the exchange rate at the time of publication of this paper, this equates to approximately $70 000 Cdn). However, the use of QALY for the measurement of cost-effectiveness is of questionable value for diseases such as IBD, which have a young patient base and low mortality rate but high morbidity[167]. Also, indirect costs are high in persons with IBD: In Sweden or Scotland, about two thirds of the costs are indirect[172,173], and in the Accent I trial of IFX, about half the patients at baseline were not employed[54]. Because of this the result of studies of cost-effectiveness and cost-utility need to be challenged, since direct costs are often not taken into consideration[174].

To quote from the report of Canadian Agency of Drugs and Technologies in Health[51], "Using cost-utility analysis cost per QALY allows for the incorporation of quality-of-life impact of clinical effects of CD treatment strategies…" and "allow for comparison with evaluation of disease area that use this outcome." The incremental cost-utility ratio for ADA and IFX, compared with usual care, was $193 305 and $451 165, respectively[51]. "….IFX and ADA have proven clinical benefit when compared to placebo, the primary and secondary non-response rates are high, the therapeutic gain is small, and…"[51] the costs associated with these treatments could be perceived as high. Furthermore, based on incremental cost utility findings from our primary economic evaluations. Adalimumab and IFX for the treatment of IBD may not be perceived to be a cost effective use of health care resources"[51] (Table 38).

CONCLUSION

The results of these and the recent meta-analyses may differ because of ".... a less contemporaneous search data, the inclusion of non-FDA-approved therapies in the analysis, and different time points for data extraction, which were not the primary end points of the included trials"[61].

In persons with luminal CD who have failed treatment with first and second-line agents or who are corticosteroid dependent, for induction of remission of active luminal CD, "IFX, natalizumab, and adalimumab appear to have the most evidence for their use, although the latter two therapies performed only moderately in this setting"[61].

"The most marked effect of biological therapies was in preventing relapse of luminal CD once remission had been achieved, with a NNT of only 4. This benefit was observed for both IFX and certolizumab, but not for adalimumab"[61].

"In terms of fistulizing CD, there is less evidence for the use of biological therapies, though there was a clear beneficial effect when only IFX was studied, although in one trial, and when only RCTs with follow-up in excess of 4 wk were considered in the analysis. There was also a benefit in favour of biological therapies over placebo when only studies that reported fistula healing in the longer term were included in the analysis."

"In preventing recrudescence of fistula, there was only one RCT reporting on this end point, which demonstrated that IFX was superior to placebo. Although concerns about adverse events, arising from the long-term use of these drugs is understandable, the data from the RCTs included in this meta-analysis did not demonstrate any increase in overall adverse events, or serious adverse events, with use for up to 1 year."

"Clinical trials for the three anti-TNFα agents approved for CD or UC have used different end point criteria and response time, in addition to the different modes of administration. Interpretations of these differences and their overall clinical relevance remain contro-

versial."

"The efficacy of anti-TNFα is limited. A total of 20%-30% of patients with refractory CD and roughly 40% of patients with refractory UC do not respond to anti-TNFα treatment and are defined as primary failures."

"Long-term therapy with biologics is associated with significant loss of response (up to 40%)."

"TNFBs represent a major advancement in the management of carefully selected patients with IBD, such as those with active fistulizing CD and active CD not responding to adequate doses of steroids and/or immunosuppressants. It is important to give these agents for an adequate duration before declaring failure of treatment. The specific time period of use is unknown, but it includes side effects to any medications or failure to achieve remission of disease."

It must be appreciated that in most TNFB studies, there is a run-in interval, and the initially approximately 30% non-responsive patients may be excluded from the find reporting of response rates; the methodology for the statistical analysis of each study must be carefully considered.

There is insufficient current evidence to support "top-down" TNFB therapy (starting early in the course of the patient's disease with TNFBs rather than the SOC progression to steroids and immunosuppressants.

The use of mucosal healing as an endpoint of successful therapy, rather than clinical response, has not been proven.

The initial response rate of fistulizing CD for induction may be higher with IFX than ADA, but longer term maintenance with ADA may be superior to IFX.

There are wide ranges in the reported rates of minor or severe adverse effects in short-term users of TNFBs. The increased risk of serious infection, lymphoma and cervical dysplasia may be as much related to the concurrent use of immunosuppressants (AZA/MTX) with TNFBs, as the TNFBs themselves. The longer term risk of TNFBs remains to be established.

It remains controversial whether immunosuppressants (AZA/MTX) should be used concurrently with TNFBs when starting TNFBs for induction therapy or continuing TNFBs for maintenance of remission. For now the international guidelines recommended using both immunomodulators and TNFB for induction of remission and mucosal healing. As well, combination therapy is preferred in moderate to severe CD as data shows reduced efficacy with single agent therapy (immunomodulators or TNFB).

It is unknown if TNFBs change the natural history of IBD.

In the UC patient who is experiencing frequent clinical recurrences while on 5-ASA, AZA or MTZ maintenance therapy, or who is hospitalized for an acute episode of their disease and fail to respond to IV steroids, it remains unclear whether IV cyclosporine followed by colectomy, AZA or IFX maintenance represent optimal therapy.

In persons with chronic continually or intermittently active UC or Crohn's colitis, it is unknown if colonoscopic surveillance protocols need to be changed to offset any potential TNFB-associated alteration in the risk of dysplasia.

When standard methods are used for economic evaluation, it is difficult to justify the use of TNFBs, based on available clinical data.

Given that some persons with IBD require TNFBs because of non-response to conventional therapies SOC, future research must solve the dilemma of predicting who will need TNFBs in the future who will be a TNFB primary or secondary non-responder, and who would benefit from early use of TNFBs. Above all, the pathogenesis of IBD needs to be identified in order than modifiable risk factors may be treated, and the diseases eventually may be prevented and cured.

The estimated 2010 expenditure on TNFBs is over $10 million (CDN), and a per IBD patient TNFB cost of $19 412 is higher than the estimated annual cost for standard of care of $17 107 for CD and $24 268 for UC.

"Making recommendations requiring TNFBs about anti-TNFα therapies based on less-than-conclusive scientific evidence has ethical implications for physicians..." (CADTH, 2009, page 60).

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REVIEW

Role for mycobacterial infection in pathogenesis of primary biliary cirrhosis?

Daniel Smyk, Eirini I Rigopoulou, Yoh Zen, Robin Daniel Abeles, Charalambos Billinis, Albert Pares, Dimitrios P Bogdanos

Daniel Smyk, Yoh Zen, Robin Daniel Abeles, Dimitrios P Bogdanos, Institute of Liver Studies and Liver Unit, King's College London School of Medicine at King's College Hospital and Kings College Hospital NHS Trust Foundation Trust, London SE5 9RS, United Kingdom
Eirini I Rigopoulou, Dimitrios P Bogdanos, Department of Medicine, University Hospital of Larissa, University of Thessaly Medical School, Biopolis, Larissa 41110, Greece
Eirini I Rigopoulou, Institute of Biomedical Research and Technology (BIOMED), CERETETH, Larissa 41222, Greece
Charalambos Billinis, Faculty of Veterinary Medicine, University of Thessaly, Karditsa 43100, Greece
Albert Pares, Liver Unit, Digestive Diseases Institute, Hospital Clínic, IDIBAPS, CIBEREHD, University of Barcelona, Barcelona 08036, Spain
Author contributions: Bogdanos DP and Rigopoulou EI conceived the idea for writing a review on this topic; Smyk D wrote the first draft of the manuscript; Smyk D and Bogdanos DP wrote the subsequent drafts of the manuscripts, although all authors contributed; Zen Y prepared and provided the histological pictures; all authors were responsible for reviewing the manuscript; all authors have read and approved the final manuscript.
Correspondence to: Dimitrios P Bogdanos, MD, PhD, Institute of Liver Studies and Liver Unit, King's College London School of Medicine at King's College Hospital and Kings College Hospital NHS Trust Foundation Trust, Denmark Hill Campus, SE5 9RS London,
United Kingdom. dimitrios.bogdanos@kcl.ac.uk
Telephone: +44-20-32993397 Fax: +44-20-32993397
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Abstract

Primary biliary cirrhosis (PBC) is a progressive cholestatic liver disease characterized by the immune-mediated destruction of biliary epithelial cells in small intrahepatic bile ducts. The disease is characterized by circulating antimitochondrial antibodies (AMAs) as well as disease-specific antinuclear antibodies, cholestatic liver function tests, and characteristic histological features, including granulomas. A variety of organisms are involved in granuloma formation, of which mycobacteria are the most commonly associated. This has led to the hypothesis that mycobacteria may be involved in the pathogenesis of PBC, along with other infectious agents. Additionally, AMAs are found in a subgroup of patients with mycobacterial infections, such as leprosy and pulmonary tuberculosis. Antibodies against species-specific mycobacterial proteins have been reported in patients with PBC, but it is not clear whether these antibodies are specific for the disease. In addition, data in support of the involvement of the role of molecular mimicry between mycobacterial and human mitochondrial antigens as triggers of cross-reactive immune responses leading to the loss of immunological tolerance, and the induction of pathological features have been published. Thus, antibodies against mycobacterial heat shock protein appear to cross-recognize AMA-specific autoantigens, but it is not clear whether these autoantibodies are mycobacterium-species-specific, and whether they are pathogenic or incidental. The view that mycobacteria are infectious triggers of PBC is intriguing, but the data provided so far are not conclusive.

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Key words: Antimitochondrial antibodies; Autoantibody; Autoimmunity; Cholestasis; Heat shock; Infection; Liver disease; Liver failure; *Mycobacterium*; Tuberculosis

Peer reviewer: Radan Bruha, MD, PhD, Associate Professor, 4th Department of Internal Medicine, General Teaching Hospital, Charles University, U Nemocnice 2, 128 08 Prague, Czech Republic

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic, autoimmune liver disease characterized by inflammatory destruction of the small intrahepatic bile ducts, fibrosis progressing to cirrhosis[1-5], and subsequent liver failure[6,7]. PBC primarily affects middle-aged women, with an increased incidence within families[2,8,9]. The prevalence of the disease is estimated to be less than 1/2000[10]. Despite the variance noted amongst countries/ethnic groups, there is a notion that the incidence of the disease is increasing[10-13].

Several autoantibody profiles have been found to be specific for the disease[14], and aid in the diagnostic workup of PBC. These include antimitochondrial antibodies (AMAs)[15-19], and/or disease-specific antinuclear antibodies (ANAs)[20-22], which are found in both symptomatic and asymptomatic patients[23]. Most common symptoms at presentation are nonspecific and include fatigue, pruritus, Sicca symptomatology and arthralgia. In more severe cases, symptoms relate to portal hypertension and hepatic decompensation (jaundice, ascites or variceal bleeding), which may indicate the need for liver transplantation[2,5,24]. The progression of PBC is generally slow and unpredictable[6,24].

The diagnosis of PBC is based on the presence of serum AMA, biochemical markers of cholestasis and histological features on liver biopsy[2]. The diagnosis of PBC is likely when at least two of these criteria are met[2,4]. Biochemical indices of cholestasis include increased levels of alkaline phosphatase and γ glutamyltransferase (GT). The diagnostic hallmark of PBC is the presence of AMA in high titers, with less than 3%-10% of patients with PBC being negative for AMA[23]. Positivity for AMA appears to be predictive of future PBC development in asymptomatic individuals[17,25]. The prevailing notion is that the presence of AMAs and their titers/concentrations do not correlate with the severity of the disease[23]. AMA seropositivity appears unable to identify patients at risk for faster progression of their liver disease compared to the seronegative cases[19,26]. Disease-specific ANAs[27-30] are also found in PBC, to a lesser degree than AMAs (20%-50% *vs* 90%-97%)[31-34], but tend to have elevated titers, and more importantly, appear to characterize patients with a more aggressive form of the disease[35-38]. Levels of IgM are also raised in most cases[2,4]. Histologically, PBC features include destruction of biliary epithelial cells and loss of small bile ducts with portal inflammatory cell infiltration, and granuloma formation (see below)[2,4,5].

The antigenic specificity of AMA[15-19] and ANA[20-22] responses have been extensively studied. PBC-specific ANAs recognize either nuclear body proteins such as the speckled protein 100 and the promyelocytic leukemia protein, or the gp210 and nucleoporin 62 nuclear membrane proteins[28,29,39-41].

AMAs in PBC are directed against the 2-oxo-acid dehydrogenase complex family of enzymes, and in particular the E2 subunits of pyruvate dehydrogenase complex (PDC-E2), branched-chain 2-oxo acid dehydrogenase complex (BCOADC) and 2-oxoglutarate dehydrogenase complex (OGDC)[2,42-44]. PDC-E2 reactivity is found in > 95% of patients with PBC, and 70% recognize BCOADC-E2 and/or OGDC-E2[2]. Reactivity to all three antigens occurs in < 50% of patients[19]. The immunodominant antigenic regions recognized by (CD4 and CD8) T lymphocytes[45-47] on PDC-E2[48-50] comprise a region within the inner lipoyl-binding domain of the subunit, spanning amino acids 212-226 (PDC-E2$_{212-226}$)[51-53]. This region is also the core target of B cell receptors, which are antibodies in their soluble form[54-56].

Medical treatment of PBC includes ursodeoxycholic acid, with the best response seen in patients who initiate treatment early in the disease[2]. These patients often show decreased or even normalized levels of alkaline phosphatase (ALP), γGT and other markers of cholestasis[2,4,6]. Studies reporting findings in large North American and European patient cohorts indicate that the percentage of patients with PBC who require liver transplantation has fallen significantly[2,57].

The cause of PBC remains undetermined[58-61], but it is believed to be the result of a genetic predisposition compounded with several lifetime exposures[62,63], similar to a "multi-hit" model[61,64-67] of disease pathogenesis[50,68,69]. Recent genome-wide association studies[70,71] have identified several HLA[72] and non-HLA[73-76] genes to be associated with PBC. Environmental factors implicated are numerous[77-81], and range from cosmetic products and xenobiotics[82], to estrogen deficiency and infectious organisms[50,58,83-85] including bacteria and viruses[50,54,58,59,63,65,66,68,69]. Mycobacteria have been included in the list of infectious organisms, partially due to the presence of granulomas in the histopathology of PBC, and the association of granulomas with mycobacteria[2,86]. In addition, AMA is found in some patients with mycobacterium-related infections[87,88]. This review will critically analyze the evidence surrounding the role of mycobacteria in the pathogenesis of PBC.

GRANULOMAS IN PBC

Granulomas consist of focal collections of inflammatory cells and cellular debris[89-92]. Their formation occurs when nondegradable products persist, as well as in hypersensitivity reactions or a combination of the two[91]. They form in a complex process involving the interaction of the infectious organism, antigen, macrophages, T cell responses, B cell hyper-reactivity, and circulating media-

Table 1 Prevalence of granulomas in liver disease *n* (%)

Ref.	Origin	Total number liver biopsies	Total number liver biopsies with granulomas	Diagnosis of PBC in granuloma group
Drebber *et al*[100]	Germany	12 161	442 (3.6)	215 (48.6)
Dourakis *et al*[101]	Greece	1768	66 (3.7)	41 (62)
Gaya *et al*[95]	United Kingdom	1662	63 (3.8)	15 (23.8)
McCluggage *et al*[96]	Northern Ireland	4075	163 (4)	90 (55)
Satti *et al*[99]	Saudi Arabia	404	59 (14.6)	Unknown

PBC: Primary biliary cirrhosis.

tors[91]. T cells involved in granuloma formation may be of the T helper (Th)1 or Th2 type[91]. Several organisms may initiate granuloma formation, including *Mycobacterium*, *Yersinia*, *Toxoplasma gondii* and *Bartonella henselae*[93,94]. Zumla *et al*[91] have noted three categories of granulomatous infections: those due to well recognized organisms; those due to organisms that are detected by molecular techniques but not by conventional microbiological techniques; and those due to organisms that have not been identified, but which are suspected. *Mycobacterium tuberculosis* (*M. tuberculosis*) is the most common organism associated with granulomas, and is histologically characterized by epithelioid cells, lymphocytes, histiocytes, Langerhans giant cells and fibroblasts surrounding a core of necrotic debris[91]. The necrotic core is usually caseating, although non-caseating granulomas may also occur[91,92].

The presence of granulomas in liver biopsies has been well documented, with a prevalence ranging from 2% to 15% in some studies, with geographical differences in prevalence rates (Table 1)[95-99]. A German study conducted by Drebber *et al*[100] examined 12 161 liver biopsies for the presence of granulomas, in addition to determining the etiology of the granuloma through histology, clinicopathological data, and polymerase chain reaction (PCR) for the detection of infectious organisms. Granulomas were found in 442 (3.6%) of the liver biopsies and interestingly, 215 were from PBC patients (1.8% of all biopsies, and 48.7% of all biopsies with granulomas)[100]. PCR demonstrated the presence of infectious organisms in 15 samples (3.4%), with *M. tuberculosis* being detected in three of the 15 (20%). It was not indicated whether any of the samples with positive PCR results came from PBC patients[100]. Although only 1.8% of liver biopsies contained granulomas, nearly half of these were obtained from patients with PBC. However, if mycobacterial infection with associated granuloma formation is a feature of PBC, a much higher percentage of granulomas would be expected. The preponderance of granulomas in liver biopsies from PBC patients has been demonstrated in other studies as well, which show geographical differences in prevalence rates. In a Northern Irish study, 55% of liver biopsies with granulomas were from PBC patients[96], compared to 23.8% in a United Kingdom study[95]. A Greek study indicates an underlying diagnosis of PBC or overlap syndrome in 62% of liver biopsies with granulomas, followed by viral hepatitis in 7.5%, and autoimmune hepatitis (AIH) in 6%[101]. Despite these findings, it should be noted that only a small percentage of granulomas can be attributed to mycobacteria, with the liver disease in these patients being due to infection and not to PBC. Figure 1 illustrates PBC-related liver granulomas contrasted with liver granulomas seen in conditions not directly related to PBC such as sarcoidosis and schistosomiasis.

The predominance of PBC as the underling liver disease in most cases of hepatic granulomas and the association of granulomas with mycobacteria, raise the question as to whether mycobacteria play a role in the pathogenesis of PBC[58,86,102]. If mycobacteria play a role in the pathogenesis of PBC (or at least in some cases of PBC), it would be expected that certain PBC-specific features, such as disease-specific AMA positivity, would also be present in patients with mycobacterial infections. Likewise, evidence of mycobacterial infection would be expected in PBC patients. This evidence may comprise more prevalent immune responses against mycobacterial antigens, or detectable mycobacterial DNA in liver tissues of PBC patients, compared to healthy and pathological controls. If mycobacterial infection plays a role in the pathogenesis of PBC, it is likely that it is limited to a triggering event, because PBC has been known to recur following transplantation[103-106] in up to 35% of patients[107]. The recurrence rate ranges from 21% to 37% at 10 years, to 43% at 15 years[108-111]. Immunosuppression with tacrolimus, as well as warm ischemic time, is associated with an increased risk of recurrence[108-112]. The more aggressive course of recurrent disease suggests that the pathological process involved in the development of PBC persists after transplantation[113]. This does not, however, suggest that the triggers of PBC also persist, and may indicate their transient involvement in the pathogenesis of PBC[113]. Neuberger highlights that hepatitis A virus can trigger autoimmune hepatitis after viral clearance, and also indicates there are several similarities between the recurrence of hepatitis C virus and PBC after transplantation, suggesting a possible infectious trigger of PBC in some individuals[113]. Although these theories are plausible, no conclusive evidence exists to demonstrate an infectious agent (including mycobacteria) as the trigger of PBC.

PBC-specific features in patients with mycobacterial infection

In an analysis of sera from 28 patients with active pul-

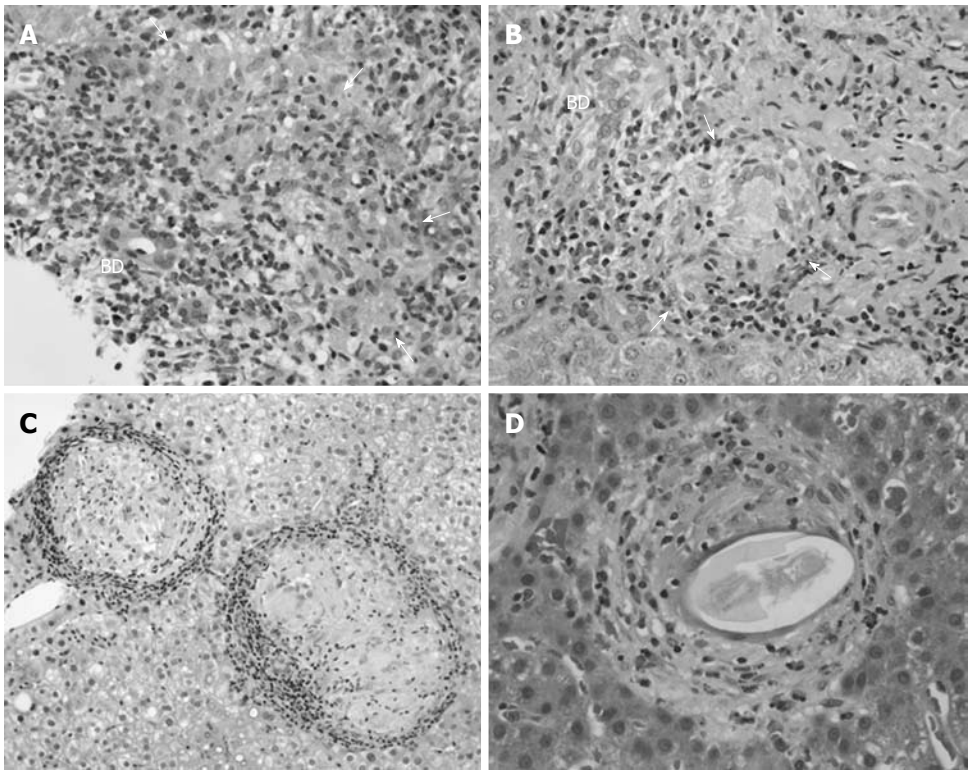


Figure 1 Epithelioid granuloma in liver biopsy. A, B: Liver specimens from patients with primary biliary cirrhosis show granuloma (arrows) close to bile ducts (BD) in portal tracts; C: Granuloma in sarcoidosis is more discrete and large in size; D: Granuloma associated with schistosomiasis contains a large number of eosinophils.

monary tuberculosis (*M. tuberculosis* infection), Klein and colleagues[88] found that 43% of these sera recognized PDC-E2 by immunoblotting based on purified mitochondrial fraction derived from beef heart mitochondria as an antigenic source. Only 2% of sera from individuals with other viral and bacterial infections [including 25 individuals with *Escherichia coli* (*E. coli*)] reacted with PDC-E2, and there was no reaction with sera from healthy controls[88]. The titers of anti-PDC-E2 antibodies were low, with no seropositive cases showing immunofixation of the 70-kDa band corresponding to PDC-E2, at a dilution of < 1/500[88]. Of relevance, none of the anti-PDC-E2 antibody-positive sera (1/10 dilution) gave an immunofluorescent pattern typical of PBC when tested by indirect immunofluorescence based on rat kidney and stomach tissue as substrates[14,16,17,88]. Among the 12 AMA-positive cases with active tuberculosis, six had abnormal levels of γGT and four had slightly elevated levels of alkaline phosphatase; only two of the cases had increased IgM levels[88]. Two of the 12 cases had alcoholic disease and of the remaining 10, none had clinical evidence of chronic liver disease[88].

A variety of autoantibodies have also been reported in the sera of patients with leprosy [caused by *Mycobacterium leprae* (*M. leprae*)], including antibodies against mitochondrial antigens[87,114,115]. In one study, the most common autoantibodies found in leprosy patients were antibodies to SS-B and mitochondria, including cardiolipin[115]. Despite the presence of AMA in the sera of leprosy patients, very few develop liver disease. Gilburd and colleagues[87] screened 69 leprosy patients with no clinical or biochemical evidence of liver disease, for the presence of anti-PDC antibodies. Positive controls consisted of three PBC patients and 18 healthy individuals served as negative controls[87]. Twenty-seven (39%) patients were found to have elevated anti-PDC antibodies by enzyme linked immunosorbent assay, but the absorbance values were relatively low; none of the normal controls had detectable AMA reactivity[87]. By immunoblotting, AMA reactivity was directed against the 54-, 41- and 35-kDa PDC subunits, and only two leprosy patients reacted with PDC-E2[87]. Inhibition studies showed that in contrast to PBC sera, none of the AMA-positive sera gave significant PDC enzyme inhibition; the rate of inhibition being similar to that noted in normal controls. Immunofluorescence testing was not performed[87].

Although AMAs are found in patients infected with mycobacteria, the reactivity patterns observed differ, with *M. tuberculosis*-infected patients showing reactivity to PDC-E2, which has not been observed in those infected with *M. leprae*[87,88]. The source of the AMAs in either case remains unknown. Gilburd *et al*[87] have suggested that the AMAs seen in leprosy patients may arise from the presence of bacterial antigens (such as *M. leprae*), which share sequence homology with the 35-, 41- and 54-kDa subunits. It is not clear whether all bands correspond to PDC subunits (such as PDC-E1 α and β) but may indeed be contaminants of other 2-oxo-acid dehydroge-

nase complexes (formerly known as M2 antigen) such as BCOADC-E2 and OGDC-E2, but reactivity to these antigens has not been tested[87].

Evidence of mycobacterial infection in PBC

In the search for evidence of antimycobacterial antibody reactivity in patients with PBC, Vilagut *et al*[86] investigated a cohort of 19 PBC patients from Spain, using membrane extracts from 10 atypical mycobacteria and found that all cases specifically reacted with *Mycobacterium gordonae* (*M. gordonae*) antigens of approximately 65 kDa and 55 kDa. It remained elusive why the other nine atypical mycobacteria were not targets of antibody responses. The unreactive mycobacterial membranes were those prepared from *Mycobacterium chelonei*, *Mycobacterium flavescens*, *Mycobacterium fortuitum* (*M. fortuitum*), *Mycobacterium intracellulare*, *Mycobacterium kansasii* (*M. kansasii*), *Mycobacterium malmoense* (*M. malmoense*), *Mycobacterium scrofulaceum*, *Mycobacterium xenopi* (*M. xenopi*), and *Mycobacterium terrae*[86]. The extraction of the 10 atypical mycobacterial membranes was performed using the same protocol, therefore, Vilagut *et al*[86] considered that their findings were not a consequence of methodological problems and suggested a biological significance for the role of *M. gordonae* in the pathogenesis of PBC.

This intriguing finding has initiated a series of subsequent studies from the same investigators as well as from independent groups[116-120]. Following the original Spanish report, a study was conducted at King's College Hospital in London investigating reactivity to *M. gordonae* and seven other atypical mycobacteria[119]. O'Donohue *et al*[119] found that 23 of 26 (88%) PBC sera reacted with a 65-kDa protein in extracts of six of the mycobacterial species tested (*M. gordonae*, *M. kansasii*, *M. fortuitum*, *Mycobacterium chelonae*, *Mycobacterium szulgai* and *M. malmoense*). Also, 15 and nine of these samples reacted with the 65-kDa band in membrane extracts of *Mycobacterium avium-intracellulare* and *M. xenopi*, respectively. However, the antibody reactivity to *M. gordonae* or other atypical mycobacteria was not restricted to PBC, but was also present in a similar prevalence in patients with other chronic liver diseases as well as in normal controls[119].

Several studies have attempted to provide evidence of mycobacterial infection in liver tissues from PBC patients. Broomé and colleagues[121] conducted an immunohistochemical study investigating liver biopsy specimens from 10 PBC cases, 13 from primary sclerosing cholangitis, five chronic hepatitis C, four alcoholic liver disease, and six healthy controls. Samples were studied using a monoclonal antibody specific for mycobacterial heat shock protein (hsp) 65. Positive staining was observed in nine of the 10 PBC cases, all primary sclerosing cholangitis, three chronic hepatitis C, and three alcoholic liver disease cases, but in none of the healthy controls[121]. Both interlobular and septal bile duct staining was observed in the PBC cases, with perinuclear staining in all positive interlobular ductal cells, and perinuclear as well as apical staining in the positive septal cells[121].

Direct evidence of microbial products in an affected tissue is best studied at the molecular level using PCR techniques. Vilagut *et al*[116] assessed the presence of *M. gordonae* DNA in liver tissue from PBC patients and controls using PCR based on amplification of a 565-bp fragment of mycobacterial gene coding for 16S rRNA on M. *gordonae*. They detected *M. gordonae* in nine of 11 (82%) PBC liver tissues but in none of the six control livers from patients with other liver diseases[116]. Contradictory results were obtained by O'Donohue *et al*[118] who investigated whether mycobacterial DNA could be detected in archival liver biopsy material from PBC and AIH. Archival material was obtained from 11 PBC and 11 AIH cases, with five lymph nodes from patients with tuberculous lymphadenopathy acting as positive controls[118]. Three of the positive controls also had liver biopsies taken for concurrent tuberculous hepatitis. No mycobacterial DNA was detected in PBC or AIH cases, while four of the five positive controls had detectable mycobacterial DNA[118]. A similar study also utilized a PCR approach for the detection of mycobacterial and other organisms in archived (paraffin embedded) liver tissues from 29 cases of PBC, as well as pathological and healthy controls. Again, no mycobacterial DNA was detected in PBC samples, and only *Helicobacter pylori* DNA was found in one case of PBC[122].

Mycobacteria and PBC: The role of molecular mimicry

The above studies do raise doubt as to whether mycobacteria play a role in PBC, although it is unclear whether a negative detection test only implies that there is no current active infection or can rule out previous infection as well. However, it has been suggested that mycobacteria and other infectious agents may not be actively present in PBC patients, but rather involved in the initiation of autoimmunity by microbial/self immunological cross-reactivity[55,56,123,124]. We and others have studied the role of molecular mimicry[68,69,123,125-128] and immunological cross-reactivity[129-133] as a mechanism responsible for the induction of autoantigen-specific immune responses in viral-hepatitis-triggered autoimmunity and several autoimmune diseases (including those affecting the liver)[134-138] in susceptible individuals[139,140]. Impairments in T-regulatory functions also appear to be a feature[141,142]. Cross-reactivity between PBC-specific mitochondrial antigens and mycobacterial proteins has been investigated by two groups[86,117]. Vilagut *et al*[86] have found that antibody responses to the 65-kDa *M. gordonae* antigen cross-reacted with anti-PDC-E2 antibodies. The same group of investigators went on to demonstrate that the 65-kDa protein was the mycobacterial hsp65, and that preincubation with PBC sera prevented binding of antibodies against hsp65[117]. The apparent cause of this cross-reactivity remained elusive until another group noted a sequence similarity between human PDC-E2 and *M. gordonae* hsp65[120]. These authors have found that the hexameric motif [GDL(IL)AE)] is shared by *M. gordonae* hsp65$_{94\text{-}99}$ and the major PBC-specific mitochondrial autoepitopes,

Table 2 Summary of studies in support or against a role of mycobacteria as triggers of primary biliary cirrhosis

Ref.	Year	Mycobacterium	M2 antigen	In support	Against
Klein *et al*[88]	1993	*M. tuberculosis*	Bovine heart	Anti-M2 AMAs (70 kDa) found in 12/28 (43%) tuberculosis patients	None of the patients had PBC AMA by IIFL were negative Anti-PDC-E2 titers were low
Gilburd *et al*[87]	1994	*M. leprae*	Bovine heart	Anti-M2 against 54-, 41- and 35-kDa bands found in 27/69 (39.1%) leprosy patients	None of the patients had PBC Only 2/27 (1%) sera reacted with the PDC-E2 band Anti-M2 inhibited anti-PDC activity in 19.1%
Vilagut *et al*[86]	1994	*M. gordonae*	Porcine heart	Anti-hsp65 *M. gordonae* antibodies found in 19/19 (100%) of PBC patients. No reactivity to other atypical mycobacteria was found Anti-porcine AMAs (anti-PDC-E2 and BCOADC-E2) cross-reacted with anti-hsp65 *M. gordonae* antibodies and *vice versa*	
O' Donohue *et al*[119]	1994	*M. gordonae*, *M. kansasii*, *M. fortuitum*, *M. chelonae*, *M. szulgai and M. malmoense*, *M. avium-intracellulare and M. xenopi*		23 of 26 (88%) PBC sera reacted with a 65-kDa protein in extracts of six of the mycobacterial species tested Sera from 15 PBC patients reacted with *M. avium-intracellulare* Sera from 9 PBC patients reacted with *M. xenopi*	Reactivity to the atypical mycobacterial mimics was found in non-PBC liver disease patients and controls
Bogdanos *et al*[54]	2004	*M. gordonae*	Primate liver	The motif GDL(IL)AE is shared by *M. gordonae* hsp65$_{94-99}$ and PDC-E2$_{216-221}$	Reactivity to *M. gordonae* hsp65$_{90-104}$ /human PDC-E2$_{212-226}$ seen in Spanish (47.5%), but rarely British patients (4%)
Broome *et al*[121]	1993	-	-	Immunohistochemistry demonstrated positive mycobacterial hsp65 staining in PBC liver tissue	Positive staining was also observed in pathological controls
Vilagut *et al*[116]	1996	*M. gordonae*	-	*M. gordonae* genetic material detected in 9 of 11 (82%) PBC liver tissues	
O'Donohue *et al*[118]	1998	*M. gordonae*	-		Mycobacterial DNA not detected in PBC liver tissues, but was detected in positive controls
Tanaka *et al*[122]	1999	Mycobacterial genus specific primers	-		Mycobacterial DNA not detected in PBC liver tissues

M. tuberculosis: *Mycobacterium tuberculosis*; *M. leprae*: *Mycobacterium leprae*; *M. gordonae*: *Mycobacterium gordonae*; *M. kansasii*: *Mycobacterium kansasii*; *M. fortuitum*: *Mycobacterium fortuitum*; *M. chelonae*: *Mycobacterium chelonae*; *M. szulgai*: *Mycobacterium szulgai*; *M. malmoense*: *Mycobacterium malmoense*; *M. avium-intracellulare*: *Mycobacterium avium-intracellulare*; *M. xenopi*: *Mycobacterium xenopi*; IIFL: Indirect immunofluorescence; PDC: Pyruvate dehydrogenase complex; PBC: Primary biliary cirrhosis; AMAs: Antimitochondrial antibodies; hsp: Heat shock protein.

namely, the inner lipoyl PDC-E2$_{216-221}$ and the outer lipoyl domain human PDC-E2$_{102-107}$[120]. No other sequences were found to be in common with *M. gordonae* hsp65 and human PDC-E2[120]. A database search analysis has found that, among bacteria, the motif SxGDL[IL]AE is virtually unique to mycobacterial hsp, and the only human sequence containing that motif was the inner lipoyl domain of PDC-E2[120]. This excellent and almost unique match between sequences of the dominant epitope of human PDC-E2 and of mycobacterial hsp65 has led the authors to investigate whether the corresponding sequences were targets of immunological cross-reactivity specifically present in patients with PBC. That study involved testing sera from 40 Spanish and 50 British PBC patients, with antibody reactivity to *M. gordonae* hsp 65$_{90-104}$/human PDC-E2$_{212-226}$ being detected in 47.5% of Spanish PBC patients and in only 4% of British PBC patients[120]. No reactivity was observed in controls. Inhibition studies confirmed that the reactivity to the mimics was due to cross-reactivity. In addition, the affinity of anti-*M. gordonae* hsp 65$_{90-104}$ antibodies was higher than that against human PDC-E2$_{212-226}$, raising the possibility that antibody reactivity to the microbe long precedes that against the human homolog[120]. Table 2 provides a summary of the above evidence for and against the role of mycobacteria in PBC.

It remains unclear whether the differing reactivity patterns noted among PBC patients from differing geographical locations (highly prevalent in Spanish PBC cases, practically absent in British PBC cases), could be explained by differing prevalence exposure rates of certain atypical mycobacteria in geographical locations. It should also be noted that multinational epidemiological studies on PBC have not indicated an association with mycobacterial infection, in contrast to what has been reported for *E. coli* or other microbes[77,78,80,81].

CONCLUSION

The presence of granulomas in liver biopsies of PBC patients raises the suspicion that bacterial infections are involved in the pathogenesis of PBC. Although infection with several bacterial species is associated with granulomatous disease, mycobacteria are the most common culprits. It is therefore not surprising that mycobacteria have been added to the list of infectious agents implicated in the pathogenesis of PBC. Furthermore, it has been observed that AMAs, which are characteristic of PBC, are also found in many patients with mycobacterial infections but their titers are low, their epitope specificity differs from that seen in PBC, and they do not appear to be detectable by conventional immunofluorescence. Additionally, mycobacteria have not been found in any significant proportion of hepatic granulomas associated with PBC. When found, the liver disease is often attributed to infection and not PBC.

It is unlikely that ongoing mycobacterial infection is a characteristic of PBC, but an early, transient infection with immunological clearance may be capable of inducing cross-reactivity. Genetic studies on PBC are now demonstrating a genetic susceptibility to the disease, and it is likely that a variety of other factors act in an additive fashion towards PBC development. These factors probably not only vary from patient to patient, but also from one geographical location to the next. A correlation between PBC and certain organisms in the context of their geographical prevalence is warranted. In addition, sequence homology between PDC-E2 and mycobacterial epitopes at the T-cell level has not been fully explored, which is of interest given that differing reactivity patterns have been observed between a variety of mycobacteria and the major mitochondrial epitope of PBC. Experimental studies in animal models of PBC involving mycobacteria may provide useful hints as to whether mycobacteria play a role in the induction of PBC. They may also distinguish between mycobacteria being a trigger of the disease, or being epiphenomena secondary to an increased susceptibility to infection in PBC patients.

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ORIGINAL ARTICLE

Indomethacin but not a selective cyclooxygenase-2 inhibitor inhibits esophageal adenocarcinogenesis in rats

Paula Esquivias, Antonio Morandeira, Alfredo Escartín, Carmelo Cebrián, Sonia Santander, Francisco Esteva, María Asunción García-González, Javier Ortego, Angel Lanas, Elena Piazuelo

Paula Esquivias, María Asunción García-González, Javier Ortego, Angel Lanas, Elena Piazuelo, CIBER Digestive and Hepatic Deseases, 08036 Barcelona, Spain
Antonio Morandeira, Alfredo Escartín, Service of Surgery, University Hospital "Lozano Blesa", 50009 Zaragoza, Spain
Carmelo Cebrián, Javier Ortego, Service of Pathology, University Hospital "Lozano Blesa", 50009 Zaragoza, Spain
Sonia Santander, Angel Lanas, Department of Medicine, University of Zaragoza, 50009 Zaragoza, Spain
Francisco Esteva, Service of Clinical Biochemistry, University Hospital "Lozano Blesa", 50009 Zaragoza, Spain
María Asunción García-González, Angel Lanas, Elena Piazuelo, Aragon Health Science Institute, 50009 Zaragoza, Spain
Angel Lanas, Service of Gastroenterology, University Hospital "Lozano Blesa", 50009 Zaragoza, Spain
Author contributions: Esquivias P wrote the manuscript; Morandeira A and Escartín A carried out all surgical procedures; Cebrián C and Ortego J performed histological analysis; Santander S, García-González MA and Esteva F were involved in all biochemical determinations and provided vital reagents and analytical tools; Lanas A and Piazuelo E were involved in the design of the study, statistical analysis and editing the manuscript.
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Correspondence to: Paula Esquivias, BSc, CIBER Digestive and Hepatic Deseases, Cardenal Goma s/n, 08036 Barcelona, Spain. pauesquivias@gmail.com
Telephone: +34-652-960252 Fax: +34-976-765478
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Abstract

AIM: To evaluate the effects of indomethacin [dual cyclooxygenase (COX)-1/COX-2 inhibitor] and 3-(3,4-difluorophenyl)-4-(4-(methylsulfonyl) phenyl)-2-(5H)-furanone (MF-tricyclic) (COX-2 selective inhibitor) in a rat experimental model of Barrett's esophagus and esophageal adenocarcinoma.

METHODS: A total of 112 surviving post-surgery rats were randomly divided into three groups: the control group (n = 48), which did not receive any treatment; the indomethacin group (n = 32), which were given 2 mg/kg per day of the COX-1/COX-2 inhibitor; and the MF-tricyclic group (n = 32), which received 10 mg/kg per day of the selective COX-2 inhibitor. Randomly selected rats were killed either 8 wk or 16 wk after surgery. The timing of the deaths was in accordance with a previous study performed in our group. Only rats that were killed at the times designated by the protocol were included in the study. We then assessed the histology and prostaglandin E2 (PGE2) expression levels in the rat esophagi. An additional group of eight animals that did not undergo esophagojejunostomy were included in order to obtain normal esophageal tissue as a control.

RESULTS: Compared to a control group with no treatment (vehicle-treated rats), indomethacin treatment was associated with decreases in ulcerated esophageal mucosa (16% *vs* 35% and 14% *vs* 17%, 2 mo and 4 mo after surgery, respectively; P = 0.021), length of intestinal metaplasia in continuity with anastomosis (2 ± 1.17 mm *vs* 2.29 ± 0.75 mm and 1.25 ± 0.42 mm *vs* 3.5 ± 1.54 mm, 2 mo and 4 mo after surgery, respectively; P = 0.007), presence of intestinal metaplasia beyond anastomosis (20% *vs* 71.4% and 0% *vs* 60%, 2 mo and 4 mo after surgery, respectively; P = 0.009), severity of dysplasia (0% *vs* 71.4% and 20% *vs* 85.7% high-grade dysplasia, 2 mo and 4 mo after surgery, respectively; P = 0.002), and adenocarcinoma incidence (0% *vs* 57.1% and 0% *vs* 60%, 2 mo and 4 mo after surgery, respectively; P < 0.0001). Treatment with the selective COX-2 inhibitor, MF-tricyclic, did not prevent development of intestinal metaplasia or adenocarcinoma. In parallel, we observed a significant decrease in PGE2 levels in indomethacin-treated rats, but not in those treated with MF-tricyclic, at both 2 mo and 4 mo. Compared to control rats that did not undergo surgery

(68 ± 8 ng/g, P = 0.0022 Kruskal-Wallis test) there was a significant increase in PGE2 levels in the esophageal tissue of the rats that underwent surgery either 2 mo (1332 ± 656 ng/g) or 4 mo (1121 ± 1015 ng/g) after esophagojejunostomy. However, no differences were found when esophageal PGE2 levels were compared 2 mo *vs* 4 mo post-esophagojejunostomy. At both the 2- and 4-mo timepoints, we observed a significant decrease in PGE2 levels in indomethacin-treated rat esophagi compared to those in either the control or MF-tricyclic groups (P = 0.049 and P = 0.017, respectively). No differences in PGE2 levels were found when we compared levels in rats treated with MF-tricyclic to not-treated rats.

CONCLUSION: In this rat model of gastrointestinal reflux, indomethacin was associated with a decrease in the severity of esophagitis and reduced development of esophageal intestinal metaplasia and adenocarcinoma.

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Key words: Intestinal metaplasia; Esophageal adenocarcinoma; Indomethacin; MF-tricyclic

Peer reviewers: Jose Liberato Ferreira Caboclo, Professor, Department of Surgery, Medical School of Sao Jose do Rio Preto, Pizza Torre 2 Apt 91, Av Bady Bassit, SJ do Rio Preto 15025-900, Brazil; Andrew Ukleja, MD, Assistant Professor, Department of Gastroenterology, Cleveland Clinic Florida, 2950 Cleveland Clinic Blvd., Weston, FL 33331, United States

Esquivias P, Morandeira A, Escartín A, Cebrián C, Santander S, Esteva F, García-González MA, Ortego J, Lanas A, Piazuelo E. Indomethacin but not a selective cyclooxygenase-2 inhibitor inhibits esophageal adenocarcinogenesis in rats. *World J Gastroenterol* 2012; 18(35): 4866-4874 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i35/4866.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i35.4866

INTRODUCTION

The incidence of esophageal adenocarcinoma (EA) in the United States and Western Europe has risen dramatically in the last few decades[1-3]. The reasons for this substantial increase are not entirely understood, but it has been linked to an increase in the incidence of gastroesophageal reflux (GERD) and Barrett's esophagus (BE), which is a recognized risk factor for the development of EA. BE is a consequence of chronic GERD, which represents the starting point of the sequence: GERD, inflammation, BE, low-grade dysplasia, high-grade dysplasia, carcinoma *in situ*, and invasive adenocarcinoma[4]. The molecular changes in the progression from GERD to BE and then to EA are not entirely understood[5], but in the last few years, cyclooxygenase-2 (COX-2) has emerged as a key player in carcinogenesis arising from BE; therefore, it is a potential therapeutic target in the sequence leading to EA. Evidence supporting the involvement of COX-2 in this sequence includes epidemiological studies that have demonstrated a protective effect of nonsteroidal antiinflammatory drugs (NSAIDs) against EA and *in vivo* animal studies in experimental models of chronic esophagitis and EA, in which administration of COX-2 inhibitors induced a reduction in the incidence of esophageal cancer. In addition, upregulation of COX-2 has been described at both mRNA and protein levels in humans with GERD, BE, or EA[6]. COX-1, the other cyclooxygenase isoenzyme, was traditionally considered to be a constitutively expressed isoform that generates prostaglandins for normal physiological functions such as platelet aggregation, gastric mucosal protection, and renal electrolyte metabolism; it was thought not be involved in carcinogenesis. Recent evidence, however, has demonstrated that COX-1 may be important in the development of different neoplasias, because elevated levels of COX-1 expression have been found in prostate, breast, and cervical cancers[7]. Furthermore, studies in $Min^{+/-}$ mice showed that homologous disruption of either *COX-2* or *COX-1* genes is followed by a reduction in polyp formation of approximately 80%[7]. In the esophagus, the role of COX-1 in the development of EA has not been investigated extensively. In a previous study, expression of both COX-1 and COX-2 were analyzed at the mRNA level in 123 primary human esophageal adenocarcinomas; mRNA levels of both isoenzymes were increased. Interestingly, RNA expression levels of COX-1 and COX-2 correlated significantly with one other, and expression of both isoenzymes correlated with the expression of vascular endothelial growth factors A and C (VEGF-A and VEGF-C)[8]. Another study reported an increase in COX-1 expression in a rat model of esophagitis induced by acid reflux[9]. Therefore, to analyze the effects of COX inhibitors in the development of EA due to gastrointestinal reflux, we assessed the effect of a dual COX-1/COX-2 inhibitor (indomethacin) in a rat model of esophageal adenocarcinoma induced by gastroesophageal reflux, and compared it with the effect of a selective COX-2 inhibitor (3-(3,4-difluorophenyl)-4-(4-(methylsulfonyl) phenyl)-2-(5H)-furanone) (MF-tricyclic).

MATERIALS AND METHODS

Animals

All of the animal studies were carried out in the Biomedicine and Biomaterials group of the University of Zaragoza, which is officially recorded as a research establishment that practices adequate husbandry and use of all research animals in accordance with Good Laboratory Practices guidelines. All procedures were approved by the in-house Ethics Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes. Throughout the experiment, all rats were housed

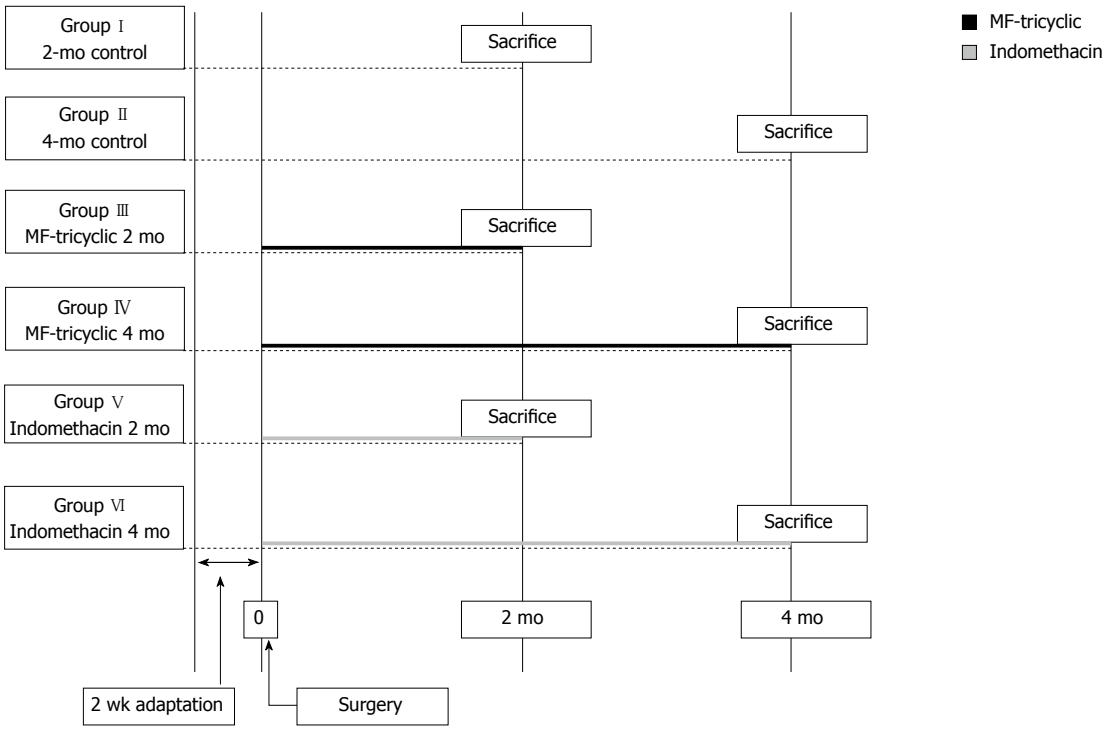


Figure 1 Scheme of the study design.

in specifically designed cages for this type of animal (EU Dim; IFFA Credo) and kept in computer-controlled conditions of light (12-h light/dark cycle), noise (45-50 dB), temperature (21 ± 2 ℃), and humidity (60%-70%). The animals were cared for by veterinary staff. A total of 128 five-week-old female Wistar rats (Harlan, Barcelona, Spain) weighing 220-300 g were used for this study.

Experimental model

Solid food was withdrawn for 24 h and water for 6-8 h before surgery. Anesthesia consisted of 75 mg/kg weight ketamine (50 mg/mL, Ketolar; Parke-Davis, Madrid, Spain) and 0.5 mg/kg weight medetomidine (1 mg/mL, Domtor; Pfizer, Madrid, Spain), which were given as a mixture in a single intramuscular injection. Before surgery, an intramuscular injection of 100 mg/kg weight cefazolin (Kefol; Lilly, Madrid, Spain) was administered as antibiotic prophylaxis. Rats underwent esophagojejunostomy with gastric preservation, allowing the gastroduodenal content to flow back into the esophagus. This surgery was performed according to a previously described protocol[10]. After surgery, the rats were housed in hanging cages to prevent bed ingestion and allowed to drink water *ad libitum*, with fasting until day 3. Water with 0.3 mg/mL buprenorphine (Buprex; Esteve, Barcelona, Spain) was provided during the first 72 postoperative hours. An additional intramuscular injection of cefazolin was administered 24 h after surgery. Rats received 50 mg/kg iron dextran (Difortin; Econatura, Madrid, Spain) intramuscularly once every 4 wk until the end of the experiment. Rats were killed by carbon dioxide inhalation either 2 mo or 4 mo after surgery. A blood sample was obtained for drug analysis from all of the animals immediately before death. Immediately after death, the esophagus was removed and opened longitudinally. Two-thirds of the esophageal specimen was prepared for pathological study. The rest of the esophagus was cut into two slices: the lower and the upper halves of the esophagus. The lower half samples were frozen immediately in liquid nitrogen and then kept at -80 ℃ until biochemical studies were performed.

General design of experiment

Two weeks after surgery, the 112 surviving post-surgery rats were randomly divided into three groups: the control group (n = 48), which did not receive any treatment; the indomethacin group (n = 32), which were given the COX-1/COX-2 inhibitor; and the MF-tricyclic group (n = 32), which received the selective COX-2 inhibitor. Randomly selected rats were killed either 8 or 16 wk after surgery. The timing of the deaths was in accordance with a previous study[10]. Only rats that were killed at the times designated by the protocol were included in the study. A scheme of the study design is shown in Figure 1. An additional group of eight animals that did not undergo esophagojejunostomy were included in order to obtain normal esophageal tissue.

Drug administration and dosage selection

MF-tricyclic was administered at a dose of 10 mg/kg body weight/d. This dose was selected according to a previous study of esophageal adenocarcinoma prevention in the same model[11]. The drug was kindly provided by Merck Frost (Quebec, Canada), which produced a pre-

mixed chow containing the drug. The concentration of MF-tricyclic in the chow was calculated using the average weight of Wistar rats and a daily consumption of 16-20 g of chow/d. This rate of chow consumption was evaluated in previous experiments[10]. Indomethacin (Sigma, Madrid, Spain) was given at a dose of 2 mg/kg body weight/d. This dose was selected according to a previous study of the inhibition of colorectal cancer[12]. The drug was added to drinking water. Indomethacin was replaced on a daily basis and administered in dark bottles in order to avoid light degradation.

Histopathological analysis

Rat esophagi were examined by two independent pathologists who were unaware of the experimental conditions. Changes in the squamous epithelium were classified into the following five categories: (1) Reactive changes: characterized by the presence of basal cell hyperplasia, increased length of papillae, and hyperkeratosis with areas of inflammation and ulceration; (2) Columnar-lined metaplasia (intestinal metaplasia): squamous epithelium was replaced with columnar-lined epithelium comprising occasional and incomplete differentiation of goblet cells; the length of the epithelial transformation was greater than 3 mm starting from the anastomosis site or was intercalated, or it could be seen beyond the surgical anastomosis, and was surrounded by squamous epithelium; (3) Dysplasia: characterized by nuclear atypia, partial loss of mucosecretory function and cell polarity, and an increase in mitotic figures. Dysplasia was classified as low-grade dysplasia (LGD) and high-grade dysplasia (HGD) according to the criteria proposed by Haggitt[13], as previously described in this experimental model[14]. In LGD, the crypt architecture is preserved or minimally distorted; the nuclei may be stratified but without reaching the apical surface of glands, nuclei are enlarged, crowded and hyperchromatic, mitotic figures may be found in the upper portion of the crypt; goblet and columnar cell mucus is usually diminished or absent, but goblet cells in which the mucous droplet does not communicate with the luminal surface may be observed. The abnormalities extend to the mucosal surface. In HGD, distortion of crypt architecture usually is present and may be marked. It is composed of branching and lateral budding of crypts, a villiform configuration of the mucosal surface or intraglandular bridging of epithelium to form a cribriform pattern of "back-to-back" glands. Nuclear abnormalities are present as in LGD, and stratification reaches the crypt luminal surface. There may be a loss of nuclear polarity, and nuclei often vary markedly in size, shape, and staining characteristics. Goblet and columnar cell mucus is usually absent. The abnormalities extend to the mucosal surface; (4) Adenocarcinoma: glandular structures of dysplastic columnar epithelial with stromal invasion and deep infiltration; and (5) Squamous carcinoma: characterized by accumulation of atypical cells with nuclear hyperchromasia, abnormally clumped chromatin, and loss of polarity. The two pathologists showed a high concordance (kappa coefficient > 0.8). However, in cases where there was disagreement, a third pathologist was required to resolve the discrepancy.

Measurement of prostaglandin E2 levels in esophageal mucosa

Prostaglandin E2 (PGE2) analysis was performed in the lower half of the esophagus. Tissue samples were added to 1 mL of cold PBS containing 10 μmol indomethacin. They were then homogenized (DIAX 900, Heidolph, Kelheim, Germany) on ice and centrifuged for 15 min at 1200 rpm (4 ℃). Supernatants were collected in 2 mL tubes. Prostaglandin extraction was performed as previously described by Powell[15]. PGE2 was measured by radioimmunoassay [prostaglandin E2 (I125); Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom].

Plasma drug levels

MF-tricyclic concentration analysis in rat plasma was performed by Merck Frost Canada; the results were disclosed to us after the study was complete. Indomethacin levels were determined as follows: 50 μg (10 μL of a 5 mg/mL methanolic solution) of the internal standard probenecid was added to 1 mL of serum before each analysis. Afterwards, samples were extracted with 2 mL of ethyl acetate and an aliquot of the organic phase was withdrawn, evaporated to dryness under a nitrogen stream at room temperature, and redissolved in 300 μL of methanol. Fifty microliters of these solutions were analyzed by high-performance liquid chromatography (Waters 600 Controller, 996 Photodiode Array Detector). A method capable of quantifying indomethacin was developed using a C-18 column (150 mm × 3.9 mm, 4 μm particle size; Waters, Barcelona, Spain) and probenecid as the internal standard. The column was equilibrated at a flow rate of 1 mL/min with a mobile phase consisting of acetonitrile in water (51:49, v/v) and 0.1% trifluoroacetic acid. The eluate was monitored at 257 nm[16].

Statistical analysis

Data management and statistical analysis were performed using SPSS software. Results from biochemical assays were expressed as mean ± SE. Data were compared between groups by nonparametric tests (Kruskall-Wallis and Mann-Whitney); $P < 0.05$ was considered to be statistically significant. Covariance analysis was used to analyze the quantitative variables of the drug, introducing the number of months as a covariant. Multiple comparisons were corrected by means of the Bonferroni method. Logistic models were adjusted for qualitative variables, evaluating in turn the effect of the drug and the number of months.

RESULTS

Macroscopically, all of the rats that underwent esophagojejunostomy showed dilatation and thickening of the middle and lower esophagus. The distal esophagus

Table 1 Macroscopic results

Group	I (*n* = 14)	II (*n* = 15)	III (*n* = 11)	IV (*n* = 6)	V (*n* = 5)	VI (*n* = 6)
Inflammation	14	15	11	6	5	6
Ulcerated mucosa,%	35.7	37.3	33.18	39.58	16	14.17
Squamous hyperplasia,%	10	14	2	5	2	2
Intestinal metaplasia in continuity with anastomosis, mm	2.29	3.5	2.32	2.83	2	1.25
Intestinal metaplasia beyond anastomosis,%	10	9	5	4	1	0
Dysplasia	14	14	10	6	5	5
High-grade dysplasia/low-to-medium grade dysplasia	10/4	12/2	4/6	4/2	0/5	1/4
Neoplasia	8	9	4	3	-	-
Mucinous adenocarcinoma	7	9	3	2	-	-
Squamous adenocarcinoma	1	-	1	1	-	-

showed ulceration. Macroscopically, we did not identify any esophageal tumors. The esophagi of all of the rats that were operated on presented inflammatory infiltrate. In most of the animals, the upper and middle parts of the esophagus showed squamous hyperplasia. The esophagi of all of the rats that were operated had columnar-lined metaplasia in continuity with the jejunal epithelium. Dysplasia had developed in all of the rats that underwent surgery. Neoplastic transformation of the epithelium was always found near the anastomosis, surrounded by columnar epithelium. Histologically, 87.5% of tumors were adenocarcinomas, and 12.5% were squamous carcinoma (Table 1). Microscopically, in some animals, a second type of metaplasia was found far above the anastomosis. This metaplasia contained isolated or grouped goblet cells, which formed authentic mucous glands in some cases. However, in any case, these metaplastic foci progressed to adenocarcinoma.

Effect of treatment on the occurrence of lesions

Inflammation, ulceration, intestinal metaplasia, dysplasia, and neoplasias were present in all of the rats that underwent esophagojejunostomy. When the percentage of ulcerated surface was analyzed, there was a significant decrease in the groups treated with indomethacin compared to the control and MF-tricyclic groups. The indomethacin treatment groups showed a significant reduction in the length of metaplasia; we also observed a significant decrease in the percentage of metaplasia beyond the anastomosis (Figures 2, 3A and B).

Most of the rats developed some degree of dysplasia. When we analyzed the occurrence of low-grade *vs* high-grade dysplasia, we found that the percentage of rats that developed high-grade dysplasia was significantly lower in rats treated with indomethacin compared to the MF-tricyclic and control groups (Figures 3C, D). The absence of neoplasias in the indomethacin-treated group (0% *vs* 36.4% in the MF-tricyclic group and 57.1% in the control group 2 mo after surgery, and 0% *vs* 50% in the MF-tricyclic group and 60% in the control group 4 mo after surgery), did not allow us to estimate the risk effect as odds ratio. Nevertheless, when we applied a log-linear model, we concluded that time was a nonsignificant factor in the presence of neoplasia. When we analyzed the effect of treatment, we observed a lower incidence of neoplasia in the indomethacin group ($P \leqslant 0.001$) (Figure 4).

Prostaglandin E2 levels

There was a significant increase in PGE2 levels in the esophageal tissue of the rats that underwent surgery either 2 mo (1332 ± 656 ng/g) or 4 mo (1121 ± 1 015 ng/g) after esophagojejunostomy compared to control rats that did not undergo surgery (68 ± 8 ng/g; $P = 0.0022$ Kruskal-Wallis test). However, no differences were found when esophageal PGE2 levels were compared 2 mo *vs* 4 mo post-esophagojejunostomy.

At both the 2- and 4-mo timepoints, we observed a significant decrease in PGE2 levels in indomethacin-treated rat esophagi compared to those in either the control or MF-tricyclic groups ($P = 0.049$ and $P = 0.017$, respectively). Rats treated with MF-tricyclic did not show differences in PGE2 levels compared to non-treated animals (Table 2).

Serum drugs levels

All of the rats treated with either MF-tricyclic or indomethacin had detectable levels of the drugs in their plasma. The serum drug concentration in rats receiving 10 mg/kg body weight MF-tricyclic was 1.62 ± 0.19 μg/mL. The rats receiving 2 mg/kg indomethacin had plasma levels of 1.97 ± 0.43 μg/mL. As expected, the rats in the untreated control group did not have detectable MF-tricyclic or indomethacin levels.

DISCUSSION

In the present study, we have used an enteroesophageal reflux model in rats to evaluate the impact of dual COX-1/COX-2 *vs* selective COX-2 inhibition in the neoplastic transformation of esophageal epithelium. Although in this experimental model the pH of the refluxate increases with increasing distance from the stomach to the anastomosis, non-acidic refluxate has been shown to enhance the occurrence of intestinal metaplasia with dysplasia and esophageal adenocarcinoma[17]. In addition, these models are widely used because they reproduce the sequence of histological changes that take place in human esophageal adenocarcinogenesis and show morphological and phenotypic characteristics similar to those observed in human Barrett's esophagus and associated

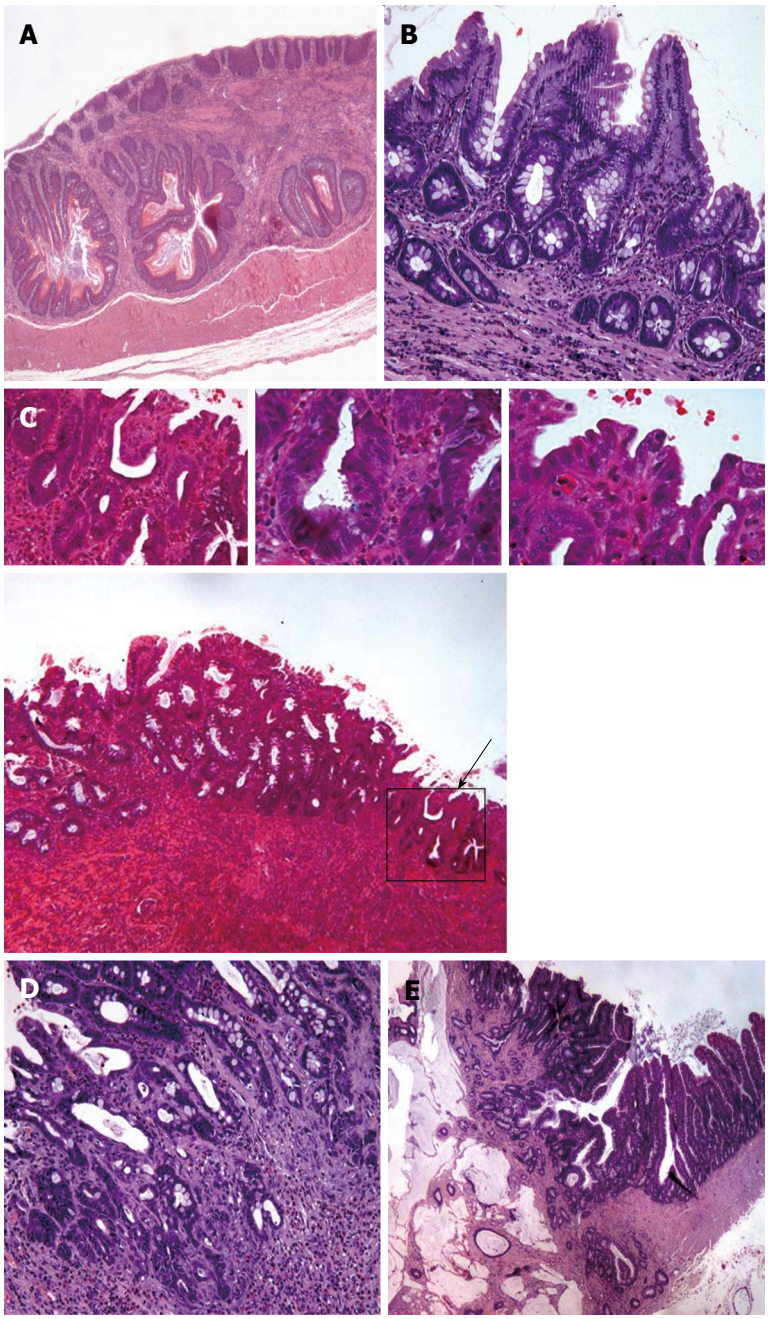


Figure 2 Pathological findings in the esophagus of rats with esophagojejunostomy. A: Hyperplasia of squamous epithelium in a control rat esophagus; B: Hematoxylin and eosin (HE) staining (× 100) showing an area of intestinal metaplasia without dysplasia. The top picture shows in more detail a gland (× 400); C: Indomethacin-treated rat. HE staining (× 40) of an area of intestinal metaplasia showing a focus with low-grade dysplasia (black arrow). The top pictures show in more detail the area with low grade dysplasia (× 200; × 400). Glands conserve their architecture. Nuclear pseudostratification can be seen, nuclear size is also increased but nuclei do not reach the lumen; D: HE staining of an area of intestinal metaplasia showing high grade dysplasia. Magnification × 100; the top picture illustrates at higher magnification (× 400), some glands with high grade dysplasia, characterized by loss of nuclear polarity and atypia; E: Photomicrograph shows an adenocarcinoma developed inside the intestinal metaplasia of the esophagus near the jejunum epithelium in a rat that had undergone an esophagojejunostomy. HE staining shows that the esophageal adenocarcinoma consists of dilated, cystic glands with abundant mucin secretion and epithelial dysplasia. (Magnification × 40; the top picture shows the same fields at × 100 magnification).

adenocarcinoma, including mucin features and expression of differentiation markers and markers of neoplastic progression[14,18].

Our results showed that treatment with the dual COX-1/COX-2 inhibitor indomethacin induced a significant reduction in the area of ulcerated mucosa, the length of metaplasia in continuity with anastomosis, the incidence of metaplasia beyond the anastomosis, the severity of dysplasia, and the incidence of adenocarcinoma in this rat model of chronic gastrointestinal reflux. In contrast, treatment with the selective COX-2 inhibitor, MF-tricyclic, did not prevent the development of these lesions in this model. Our results are consistent with those reported by Heath *et al*[19] in a human clinical trial, in which treatment with 200 mg/d of celecoxib, another selective inhibitor of COX-2, did not prevent the progression of Barrett's dysplasia to cancer. In parallel with our histopathological results, we found a significant decrease in PGE2 levels in indomethacin-treated rats, but not in the MF-tricyclic group, which suggests that PGE2 inhibition is also linked to the effect observed with these two agents. Increased levels of PGE2 during chronic inflammation were previously correlated with carcinogenesis. In an esophagitis rabbit model, Lanas *et al*[20] observed high PGE2 levels associated with increased activity of both COX-1 and COX-2. The levels decreased when rabbits were treated with the nonselective COX inhibitor, indomethacin.

In an experimental model, Buttar *et al*[11] showed that treatment with sulindac had a preventive effect against EA. In contrast with our findings, they observed a similar, although numerically lower, beneficial effect with MF-

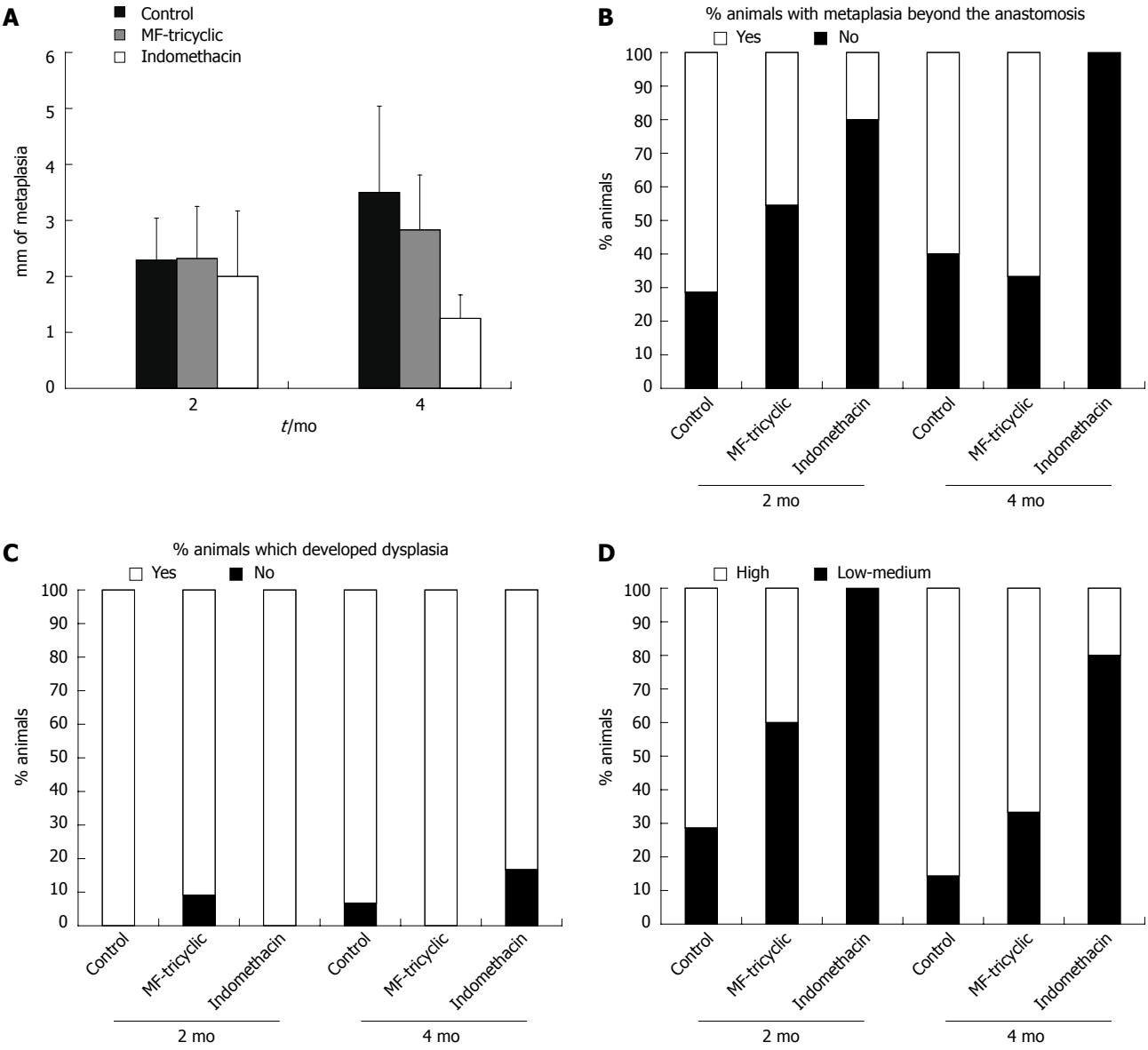


Figure 3 Macroscopic findings. A: Effect of treatment on metaplasia in continuity length. A significant decrease was observed in indomethacin-treated groups compared to control groups (P = 0.007); B: Treatment effect on the percentage of animals that developed metaplasia beyond the anastomosis. Percentage of animals that developed metaplasia beyond the anastomosis was significantly higher in both control and MF-tricyclic groups (P = 0.009 and P = 0.036, respectively); C: Percentage of animals that developed dysplasia; D: Because most animals developed dysplasia, low-to-medium grade *vs* high-grade dysplasia was analyzed. Percentage of rats that developed high-grade dysplasia was significantly lower in indomethacin groups compared to control groups (P = 0.002). When we analyzed the effect of treatment, we observed lower incidence of neoplasia in the indomethacin group (P = 0.000). MF-tricyclic: 3-(3, 4-difluorophenyl)-4-(4-(methylsulfonyl)phenyl)-2-(5H)-furanone.

tricyclic, although they also did not see significant mucosal PGE2 inhibition with this agent. In our study, we used the same dose of MF-tricyclic as that reported in Buttar's experiment, but the serum drug levels found in our rats were higher. Therefore, we suggest that the apparent differences between these results could be due to the different experimental periods of treatment (28 and 16 wk in Buttar's and our study, respectively). In a 40-wk experimental study, Oyama *et al*[21] showed that pathological changes associated with accelerated cell proliferation, such as regenerative thickening and basal cell hyperplasia, were significantly suppressed in the treated group (celecoxib 500 ppm, 100 mg/kg/d) throughout the experiment. However, the incidence of columnar lined epithelium and EA were significantly suppressed only at the end of the study (40 wk). Similarly, Chen *et al*[22] showed beneficial effects in animals treated with celecoxib over a 40-wk experimental period. We selected the endpoint of the present study according to previous research performed in our group, in which rats under esophagojejunostomy showed a high incidence of esophageal adenocarcinoma and intestinal metaplasia 4 mo after surgery. It is possible that this period of time is sufficient to see a positive effect with profound inhibition of both types of COX activity as with indomethacin, but is not long enough to see the results of weaker or partial COX inhibition with a selective COX-2 agent.

Most studies, including ours, showed a greater decr-

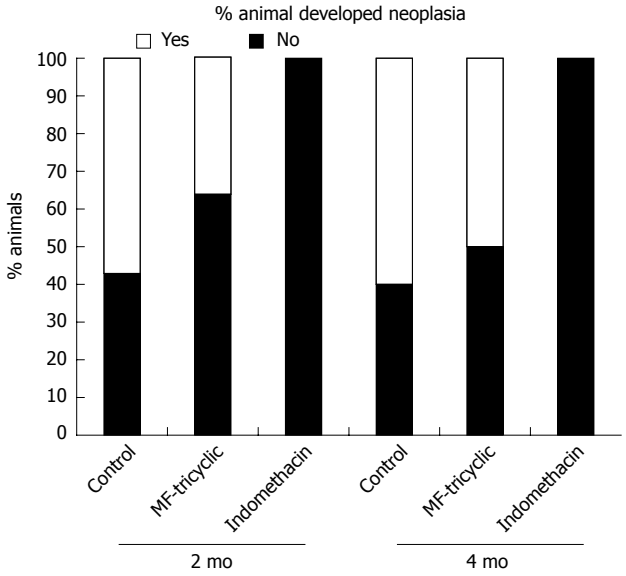


Figure 4 Percentage of animals that developed neoplasia.

ease in tissue PGE2 levels with a nonselective COX inhibitor compared to COX-2 selective inhibitors[11,20,23]. This effect suggests that PGE2-derived COX-1 inhibition may play a role in the progression from gastrointestinal reflux to EA. In addition, additional mechanisms not linked to either COX-1 or COX-2 inhibition may be involved in this process. Orido *et al*[24] observed that treatment with indomethacin, but not sulindac, decreases arachidonic acid uptake in a human colorectal cell line. They suggested that the anticancer effect of indomethacin could be independent of COX-2 inhibition. Moreover, Kim *et al*[25] evaluated the induction of NAG-1 and apoptosis by various NSAIDs, such indomethacin, sulindac, and diclofenac. Indomethacin was the most potent inducer of both NAG-1 expression ($P < 0.05$) and apoptosis ($P < 0.05$). Kopp *et al*[26] and Castro *et al*[27] reported that sodium salicylate, acetylsalicylic acid, and indomethacin inhibit activation of NF-κB[26,27]. Hanif *et al*[28] observed that NSAIDs inhibit the proliferation rate of two colon cancer cell lines independent of their ability to inhibit prostaglandin synthesis.

In addition to the above observations, it is important to note that much higher doses of NSAIDs are required for inhibition of proliferation and angiogenesis and induction of apoptosis, some of the mechanisms that have been suggested to contribute to antitumor activity, than for inhibition of prostaglandin synthesis[29].

In conclusion, treatment with indomethacin, a dual COX-1/COX-2 inhibitor, in our experimental model of EA, prevented the development of intestinal metaplasia and esophageal adenocarcinoma and reduced PGE2 production. In contrast, treatment with MF-tricyclic, a selective COX-2 inhibitor, was not associated with these effects. The profound inhibition of mucosal PGE2 with indomethacin, but not with MF-tricyclic, might partly explain the different effects, but other reasons, such as the length of therapy, activity of COX-1, and involvement of COX-independent mechanisms, could also explain the results, and deserve further investigation.

Table 2 Esophageal prostaglandin E2 levels in rats at 2 mo and 4 mo (mean ± SE, ng/g)

	2 mo	4 mo
Without treatment	1332.7 ± 656.7	1121.6 ± 1015.6
MF-tricyclic	1776.8 ± 1403.3	1914.9 ± 1905.5
Indomethacin	480.3 ± 333.4	243.9 ± 85.7

MF-tricyclic: 3-(3,4-difluorophenyl)-4-(4-(methylsulfonyl)phenyl)-2-(5H)-furanone.

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COMMENTS

Background

Over the last decades, the incidence of esophageal adenocarcinoma in the United States and Western Europe has dramatically risen. Epidemiological studies have reported that consumption of cyclooxygenase (COX) inhibitors leads to a decrease in the chance of developing esophageal cancer.

Research frontiers

COX-2 has become a target for the prevention of esophageal adenocarcinoma. There are several studies in which administration of COX-2 inhibitors showed a reduction in the incidence of esophageal adenocarcinoma in rat reflux-induced models. In contrast to previous studies, the research showed no effect when a selective COX-2 inhibitor was administered, while a non-selective COX inhibitor, indomethacin, inhibited the complete carcinogenic sequence in the rat esophagus.

Innovations and breakthroughs

Recent evidence has demonstrated that COX-1 may be important in the development of different neoplasias, because elevated levels of COX-1 expression were found in prostate, breast, and cervical cancers. In the study, treatment with indomethacin, a dual COX-1/COX-2 inhibitor, prevented the development of intestinal metaplasia and esophageal adenocarcinoma and reduced prostaglandin E2 (PGE2) production. In contrast, treatment with 3-(3, 4-difluorophenyl)-4-(4-(methylsulfonyl)phenyl)-2-(5H)-furanone (MF-tricyclic), a selective COX-2 inhibitor, was not associated with these effects. These results suggest that COX-1 also plays an important role in the development of esophageal adenocarcinoma.

Applications

The inhibitory effect of the complete carcinogenic sequence observed with indomethacin in this study suggests two new aspects that should be further investigated; firstly, the potential role of COX-1 in esophageal adenocarcinogenesis and secondly, the involvement of cyclooxygenase-independent anticancer actions of indomethacin.

Peer review

The authors evaluated the effect of indomethacin, a COX non-selective inhibitor, and MF-tricyclic, a COX-2 selective inhibitor, in an experimental model of Barrett's esophagus and EA in rats. The study showed that indomethacin but not MF-tricyclic prevented the development of intestinal metaplasia and esophageal adenocarcinoma and reduced PGE2 production. The results suggest that COX-1 plays an important role in the development of esophageal adenocarcinoma.

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ORIGINAL ARTICLE

Effect of Tangweian Jianji on upper gastrointestinal remodeling in streptozotocin-induced diabetic rats

Gui-Fang Liu, Jing-Bo Zhao, Zhong Zhen, Hong Sha, Peng-Min Chen, Min Li, Jia-Cheng Zhang, Ming-Ze Yuan, Wen Gao, Hans Gregersen, Xiao-Lin Tong

Gui-Fang Liu, Zhong Zhen, Min Li, Jia-Cheng Zhang, Ming-Ze Yuan, Xiao-Lin Tong, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing 100053, China
Jing-Bo Zhao, Aalborg Hospital, DK 9000 Aalborg, and Institute of Clinical Medicine, Aarhus University, DK8200 Aarhus N, Denmark
Hong Sha, Peng-Min Chen, Institute of Clinical Medicine, China-Japan Friendship Hospital, Beijing 100029, China
Wen Gao, China Pharmaceutical University, Nanjing 211198, Jiangsu Province, China
Hans Gregersen, Sino-Danish Centre for Education and Research, DK-8000 Aarhus C, Denmark
Author contributions: Tong XL and Gregersen H contributed equally to this work; Tong XL, Gregersen H, Liu GF and Zhao JB designed the research; Liu GF, Sha H, Yuan MZ and Zhang JC performed the experiment; Chen PM and Zhen Z contributed new analytic tools; Li M performed the molecular biology detection; Gao W performed the pharmaceutical detection; Liu GF and Zhao JB analyzed the data; Liu GF and Zhao JB wrote the manuscript; Gregersen H revised the manuscript.
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Correspondence to: Xiao-Lin Tong, Professor of Medicine, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, No. 5 Beixiange St., Xuanwu District, Beijing 100053, China. xiaolintong66@sina.com
Telephone: +86-10-88001260 Fax: +86-10-88001166
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Abstract

AIM: To investigate the effect of Tangweian Jianji (TWAJJ) on the biomechanical and morphometrical remodeling of the upper gastrointestinal tract in diabetic rats.

METHODS: Diabetes was induced in 27 rats by injecting streptozotocin (40 mg/kg body weight), the animals were then divided into three groups (n = 9 in each group), i.e., diabetic control (DM); high dose (10 g/kg, T1) and low dose (5 g/kg, T2). Another 10 rats acted as normal controls (Control). TWAJJ was administered by gavage once daily. Blood glucose and serum insulin levels were measured. Circumferential length, wall thickness and opening angle were measured from esophageal, duodenal, jejunal and ileal ring segments. The residual strain was calculated from the morphometric data. Step-wise distension was carried out on esophageal and jejunal segments. The obtained data on the length, diameter and pressure changes were then used to calculate the circumferential and longitudinal stresses and strains. Real-time reverse transcription polymerase chain reaction was used to detect the receptor of advanced glycation end-products (RAGE) mRNA level in jejunal tissues.

RESULTS: At the end of the experiment, the blood glucose level was significantly higher and the serum insulin level was significantly lower in DM, T1 and T2 groups than in the control group (Glucose: 30.23 ± 0.41 mmol/L, 27.48 ± 0.27 mmol/L and 27.84 ± 0.29 mmol/L *vs* 5.05 ± 0.04 mmol/L, $P = 1.65 \times 10^{-16}$, $P = 5.89 \times 10^{-19}$ and $P = 1.63 \times 10^{-18}$, respectively; Insulin: 1.47 ± 0.32 μg/L, 2.66 ± 0.44 μg/L, 2.03 ± 0.29 μg/L and 4.17 ± 0.54 μg/L, $P = 0.0001$, $P = 0.029$ and $P = 0.025$, respectively). However, these levels did not differ among the DM, T1 and T2 groups. The wet weight per unit length, wall thickness and opening angle of esophageal and intestinal segments in the DM group were significantly higher than those in the control group (from $P = 0.009$ to $P = 0.004$). These parameters in the T1 group were significantly lower than those in the DM group (wet weight, duodenum: 0.147 ± 0.003 g/cm *vs* 0.158 ± 0.001 g/cm, $P = 0.047$; jejunum, 0.127 ± 0.003 g/cm *vs* 0.151 ± 0.002 g/cm, $P = 0.017$; ileum, 0.127 ± 0.004 g/cm *vs* 0.139 ± 0.003 g/cm, $P = 0.046$; wall thickness, esophagus: 0.84 ± 0.03 mm *vs* 0.94 ± 0.02 mm, $P = 0.014$; duodenum: 1.27 ± 0.06 mm *vs* 1.39 ± 0.05 mm, $P = 0.031$; jejunum: 1.19 ± 0.07

mm *vs* 1.34 ± 0.04 mm, *P* = 0.047; ileum: 1.09 ± 0.04 mm *vs* 1.15 ± 0.03 mm, *P* = 0.049; opening angle, esophagus: 112.2 ± 13.2° *vs* 134.7 ± 14.7°, *P* = 0.027; duodenum: 105.9 ± 12.3° *vs* 123.1 ± 13.1°, *P* = 0.046; jejunum: 90.1 ± 15.4° *vs* 115.5 ± 13.3°, *P* = 0.044; ileum: 112.9 ± 13.4° *vs* 136.1 ± 17.1°, *P* = 0.035). In the esophageal and jejunal segments, the inner residual stain was significantly smaller and the outer residual strain was larger in the DM group than in the control group (*P* = 0.022 and *P* = 0.035). T1 treatment significantly restored this biomechanical alteration (*P* = 0.011 and *P* = 0.019), but T2 treatment did not. Furthermore, the circumferential and longitudinal stiffness of the esophageal and jejunal wall increased in the DM group compared with those in the control group. T1, but not T2 treatment, significantly decreased the circumferential wall stiffness in the jejunal segment (*P* = 0.012) and longitudinal wall stiffness in the esophageal segment (*P* = 0.023). The mRNA level of RAGE was significantly decreased in the T1 group compared to that in the DM group (*P* = 0.0069).

CONCLUSION: TWAJJ (high dose) treatment partly restored the morphometric and biomechanical remodeling of the upper gastrointestinal tract in diabetic rats.

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Key words: Biomechanics and morphometric remodeling; Diabetes rats; Gastrointestinal tract; Mechanism; Tangweian Jianji

Peer reviewers: Dr. Kazuaki Takabe, MD, PhD, Surgical Oncology, Virginia Commonwealth University, West Hospital 7-402, 1200 East Broad Street, Richmond, VA 23298-0011, United States; Shivananda Nayak, PhD, Biochemistry Unit, Department of Preclinical Sciences, Faculty of Medical Sciences, The University of The West Indies, Building 36, Mount Hope EWMSC, Trinidad and Tobago; Islam Khan, Professor, Department of Biochemistry, Kuwait University, Jabrya 13110, Kuwait

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INTRODUCTION

Diabetic gastrointestinal disorder (DGID) is a common complication of diabetes. Up to 76% of diabetic patients express gastrointestinal symptoms including dysphagia, early satiety, reflux, constipation, abdominal pain, nausea, vomiting and diarrhea[1,2]. Duration of the disease and poor glycemic control seem to be associated with the severity of gastrointestinal (GI) problems. In general, the pathogenesis of DGID includes high blood glucose level, smooth muscle degeneration, abnormal autonomic neuropathy, gastrointestinal hormone secretion disorders and oxidative stress[3,4].

The GI tract is functionally subjected to dimensional changes. Hence, biomechanical properties such as the stress-strain relationships are of particular importance[5]. These properties are remodeled in response to growth[6], fasting[7] and disease[8]. The biomechanical properties are crucial for GI motor function because peristaltic motion which propels the food through the GI tract is a result of an interaction between the passive and active tissue forces and the hydrodynamic forces in the food bolus. Remodeling of the mechanical properties reflects the changes in the tissue structure that determine a specific motor dysfunction. During the past few years, several studies have demonstrated that experimental diabetes induces GI morphological and biomechanical remodeling[9-13]. Following the development of diabetes, the GI wall becomes thicker and stiffer in a time-dependent manner. Therefore, diabetic gastrointestinal morphological and biomechanical remodeling play an important role in DGID and has become the new perspective of diabetic gastrointestinal pathogenesis[11].

Some studies on diabetic arteries have demonstrated that non-enzymatic glycation of arterial wall tissues is associated with remodeling of the wall[14,15]. We believe that the same applies to the diabetic GI wall. Advanced glycation end-products (AGEs) may contribute to diabetic GI morphological and biomechanical remodeling by two major mechanisms. The first is receptor-independent alteration of the extracellular matrix architecture by non-enzymatic glycation and the formation of protein cross-links. The second mechanism is receptor-dependent and consists of modulation of cellular functions through ligation of specific cell surface receptors[16-18].

Western medical treatment of DGID is mainly focused on symptomatic control with improvement of gastric motility, using promoting agents and supportive measures based on blood glucose control[2]. Although these therapies can partially improve the clinical symptoms, they do not fundamentally reverse the diabetes-induced changes and result in a very high relapse rate. In clinics it was shown that Tangweian Jianji (TWAJJ) significantly improved DGID with a low relapse rate, however, the mechanism involved in this improvement is not fully understood. Therefore, the aim of the present study was to investigate whether TWAJJ treatment can improve the morphometric and biomechanical remodeling of the GI tract in streptozotocin (STZ)-induced diabetic rats. Furthermore, the receptor of advanced glycation end-products (RAGE) mRNA level in the jejunal tissues was also investigated to explore the possible mechanism of TWAJJ in the treatment of DGID.

MATERIALS AND METHODS

Animal model and groups

Forty male SD rats weighing 220-250 g were included in this study. Diabetes was induced in 30 rats by a single tail vein injection of 40 mg/kg body weight of strepto-

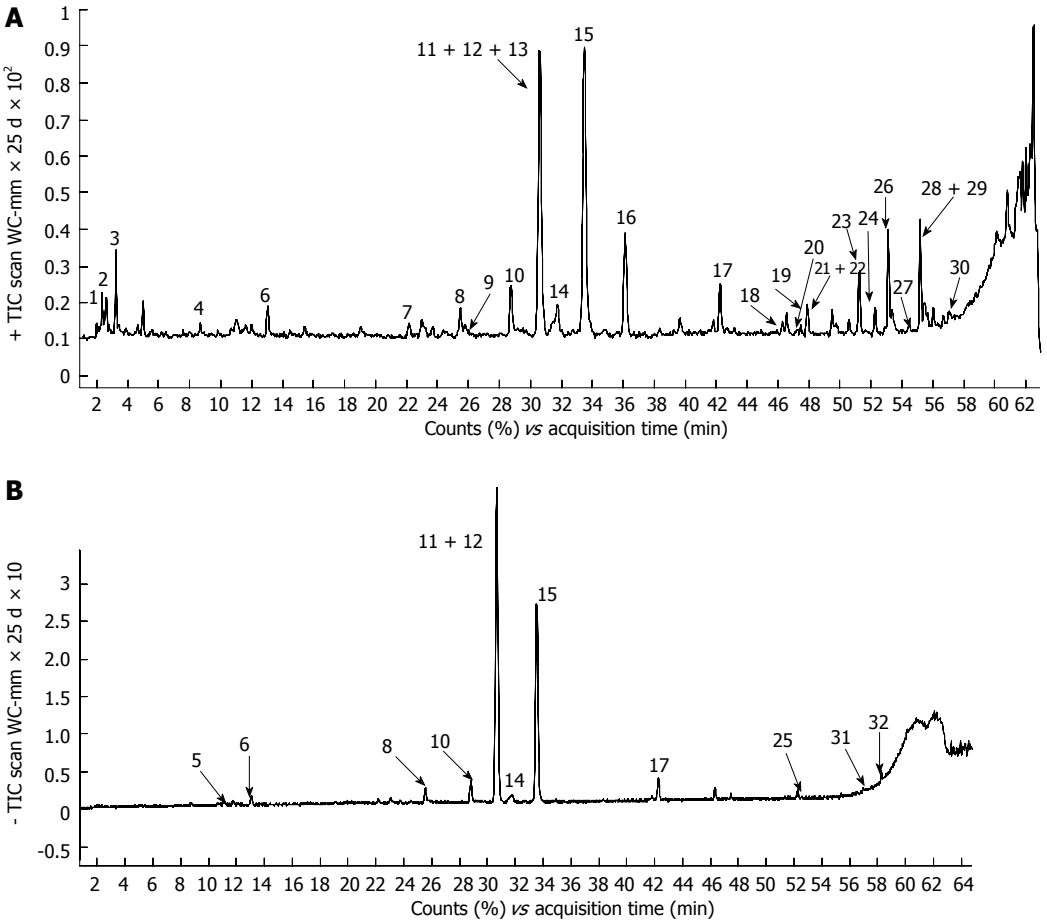


Figure 1 High pressure liquid chromatograph/mass spectrometry analysis of Tangweian Jianji extract. A: Representative total ion current (TIC) chromatograms of the Tangweian Jianji (TWAJJ) extract were obtained in positive ion electrospray; B: TIC chromatograms of the TWAJJ extract was obtained in negative ion electrospray. Visual inspection of the two TIC plots shows that the two modes of ionization generate different constituent information based on the ionizability differences of the components in TWAJJ. A total of 32 peaks were observed. The most abundant compounds identified are Naringin (peak 12), Neohesperidin (peak 15), Lobetyolin (peak 16), Atractylenolide (peak 26) and Emodin (peak 23). WC: Wave chromatogram.

zotocin (STZ, Sigma-Aldrich, China). This dose of STZ resulted in a random blood glucose level ⩾ 16.7 mmoL/L in 90% of rats 7 d after the injection. The remaining 10% of rats were excluded from this study. Twenty-seven STZ-induced diabetic rats were subdivided into three groups (n = 9 in each group), i.e., diabetic control group (DM); high dose of TWAJJ group (T1) and low dose of TWAJJ group (T2). Another 10 rats of similar age and body weight from the same vendor were used as a non-diabetic control group (Control).

Drugs and administration methods

TWAJJ is composed of Citrus aurantium, Codonopsis, fried Atractylodes and wine Rhubarb and was provided by Guang'anmen Hospital, China Academy of Chinese Medical Sciences. The medicine was administered by gavage which passed though the esophagus and reached the stomach lumen. The drug was perfused directly into the stomach once daily from the beginning of the experiment. The dosage was 10 g/kg for T1 and 5 g/kg for T2, respectively. The rats in the DM and control groups were perfused with physiological saline.

Chemical analysis of TWAJJ

The formulation of TWAJJ was prepared according to the corresponding monograph in the 2005 Edition of Chinese Pharmacopoeia. There were quantitative control limits for the raw herbs (for example, the content of Naringin in Citrus aurantium was not less than 4.0% and Neohesperidin was not less than 3.0%) and for the final drug product. The chemical composition of TWAJJ was determined by using an ultra-fast high performance liquid chromatography-electrospray-ionization-quadrupole time-of-flight mass spectrometry method. An example chromatographic fingerprint is shown in Figure 1. The major compounds in the final dosage form were identified as Naringin, Neohesperidin, Lobetyolin, Atractylenolide and Emodin. These compounds were used as the quality control markers in the final TWAJJ formulation.

Experimental procedures

Weight and blood glucose levels were measured at 2-wk intervals after initiating the experiment. For blood glucose measurement, one drop of blood was obtained from rat tail vein and blood glucose was measured by a John-

son and Johnson One Touch Ultra Blood Glucose Meter. For insulin measurement, blood was obtained from the abdominal aorta and 0.2 mL serum was separated. The serum insulin was measured by radioimmunoassay (rat insulin radioimmunoassay kit, Linco Company, United States) at the end of the experiment.

The experimental period was 60 d. At the end of the experiment, the rats were fasted overnight and anesthetized with 4% Chloral hydrate (10 mL/kg, *ip*). Following laparotomy, the whole esophagus and ten centimeters of duodenum, jejunum and ileum were harvested. The duodenum was taken from the descending section, 1 cm down from the pylorus; the jejunum from 5 cm distal to the ligament of Treitz, and the ileum from 5 cm proximal to the ileo-cecal valve. After gently cleaning the lumen of the segments with saline, the length and wet weight were measured.

The esophageal and jejunal segments were divided into three sections, the proximal 1 cm segment was immediately stored at -70 ℃ for protein and RAGE mRNA detection. The adjacent 1 cm long segment was used for the zero-stress state experiment. The remaining section was used for the distension test. The duodenal and ileal segments were divided into two and used for protein and mRNA detection and the zero-stress state experiment. In this experiment, RAGE mRNA detection was performed only on the jejunal segments.

Zero-stress state experiment

To obtain data on the zero-stress state, three 1-2 mm wide esophageal and intestinal rings were cut and placed in Krebs solution at room temperature. The composition of Krebs solution (mmol/L) was: NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; NaH_2PO_4, 1.0; $MgCl_2$, 1.2; and ascorbic acid, 0.11. A photograph was taken of the cross-section of the rings using a Canon camera (Canon, Japan) and was presented as the no-load state. Each ring-shaped segment was then cut radially from the opposite mesentery site and the photographs were taken about 60 min after the radial cutting to allow viscoelastic creep to take place. This is presented as the zero-stress state.

Distension test

The distal end of the remaining esophageal and jejunal segments was tied with a suture and the proximal end was cannulated with a tube for the distension experiment. After preconditioning of the segments, they were inflated with Krebs solution using a step-wise distension protocol from 0 cm to 20 cm H_2O (0 cm, 1 cm, 2 cm, 3 cm, 5 cm, 10 cm, 15 cm and 20 cm H_2O) for the esophageal segment and from 0 cm to 10 cm H_2O (0 cm, 0.5 cm, 1 cm, 2 cm, 4 cm, 6 cm, 8 cm and 10 cm H_2O) for the jejunal segment. The segments conformed to a cylindrical geometry during the distensions. Each pressure lasted for 2 min and then the outer diameter and length of the segments were photographed (Canon, Japan).

Mechanical data analysis

The morphometric data were obtained from digitized images of the segments in the zero-stress, no-load and pressurised states. Measurements were undertaken using image analysis software (Sigmascan ver. 4.0, Sigma Corp., San Rafael, CA, United States). The following data were measured from each specimen: the circumferential length (C), the wall thickness (h), the wall area (A), and the opening angle at zero-stress state (α). The subscripts i, o, n, z and p refer to the inner (mucosal) surface, outer (serosal) surface, no-load state, zero-stress state and pressurised condition. The opening angle α was defined as the angle subtended by two radii drawn from the midpoint of the inner wall to the inner tips of two ends of the specimen. Furthermore, the outer diameter (D) and the length (L_p) were measured from the images of the pressurised segments.

The measured data were used for the calculation of biomechanical parameters defined as:

Residual Green's strain at the mucosal surface:

$$E_i = \frac{(\frac{C_{i-n}}{C_{i-z}})^2 - 1}{2} \quad (1)$$

Residual Green's strain at the serosal surface:

$$E_o = \frac{(\frac{C_{o-n}}{C_{o-z}})^2 - 1}{2} \quad (2)$$

The stress and strain of the esophageal and intestinal segments in the pressurised state were determined under the assumptions that the wall was homogenous and the organ shape was cylindrical. Calculations were performed knowing the no-load state dimensions, the outer diameters and lengths of the specimens at varying pressures, and assuming incompressibility of the wall. The longitudinal stretch ratio, $\lambda_\varphi = \frac{L_p}{L_z}$;

the luminal radius, $r_{i-p} = \sqrt{r_{o-p}^2 - \frac{A_n}{\pi\lambda_\varphi}}$;

the wall thickness, $h_p = r_{o-p} - r_{i-p}$; the mucosal circumferential length, $C_{i-p} = 2 \times \pi \times r_{i-p}$; the serosal circumferential length, $C_{o-p} = 2 \times \pi \times r_{o-p}$; the mid-wall circumferential length, $C_{m-p} = \frac{C_{i-p} + C_{o-p}}{2}$;

the circumferential stretch ratio, $\lambda_\theta = \frac{C_{m-p}}{C_{m-z}}$

(where the middle-wall circumferential length at zero-stress state, $C_{m-z} = \frac{C_{i-z}}{C_{o-z}}$) were calculated.

Then the Kirchhoff's stress and Green's strain in a wall at a given pressure were calculated according to the following equations:

Circumferential Kirchhoff's stress: $S_\theta = \frac{\Delta P r_{i-p}}{h_p \lambda_\theta^2}$ (3)

Longitudinal Kirchhoff's stress: $S_\varphi = \frac{\Delta P r_{i-p}^2}{h_p \lambda_\varphi^2 (r_{o-p} + r_{i-p})}$ (4)

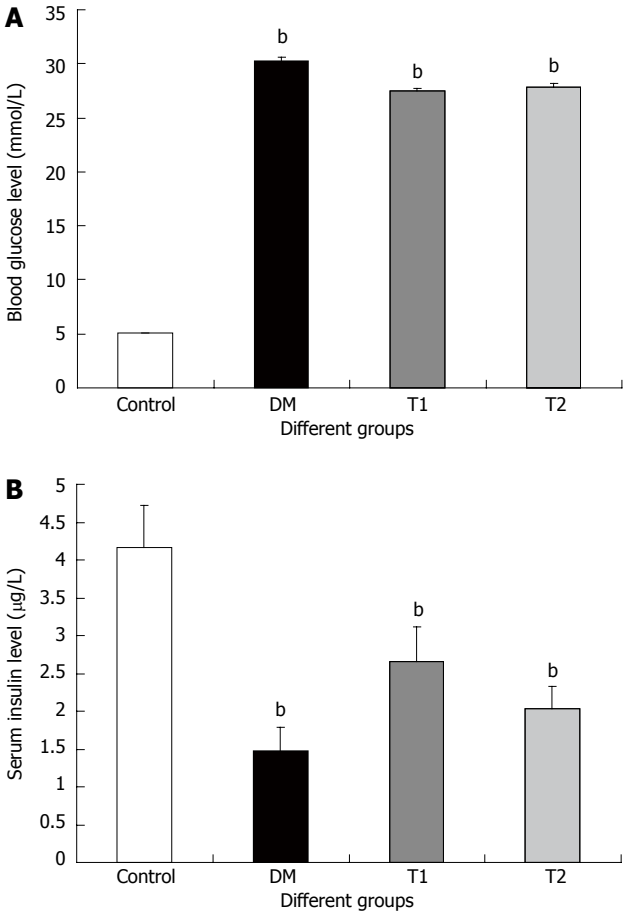


Figure 2 Blood glucose levels and serum insulin levels in the different groups. A: Blood glucose levels; B: Serum insulin level. DM, T1 and T2 groups compared with the control group. $^{b}P < 0.01$ *vs* control group. DM: Diabetic control; T1: High dose; T2: Low dose.

$$\text{Circumferential mid-wall Green's strain: } E_{\theta} = \frac{\lambda_{\theta}^{2} - 1}{2} \quad (5)$$

$$\text{Longitudinal Green's strain: } E_{\varphi} = \frac{\lambda_{\varphi}^{2} - 1}{2} \quad (6)$$

ΔP is the transmural pressure difference. The longitudinal mid-wall stretch ratio was referenced to the no-load state because tissue strips could not be cut to obtain the zero-stress state in the longitudinal direction. However, the longitudinal mid-wall length in rat intestine does not differ between the no-load and zero-stress states[19].

Receptor of advanced glycation end-products mRNA detection

Real-time polymerase chain reaction (RT-PCR) was used to detect RAGE mRNA level in the jejunal tissues. Total RNA was isolated from 100 mg tissue of each specimen using ultra-pure Trizol reagent according to the procedure described by the manufacturer (Invitrogen, United States). RNA concentration was determined when equal amounts of total RNA (3 μg) were used in the reverse transcription with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, United States). RT-PCR was used to quantify the mRNA expression of RAGE. Amplification was performed using iQ Supermix with the iCycler iQ Real-Time Detection System according to the manufacturer's instructions (Applied Biosystems). A standard curve was generated for each primer pair and a probe to determine PCR efficiency. Mean threshold cycle (Ct) values for each sample were normalized to β-actin and calibrated to the control group to obtain the threshold cycle difference (ΔΔCt) with 2-ΔΔCt being the fold change relative to the control group. The probes and primer sequences used were as follows: RAGE: Sense: 5'-TCT CAG AAG CCC AAG GAA GAG T-3'; Antisense: 5'-CCT AGG TCT GAA GGC CCT GAG T-3', amplified cDNA length: 173 bp; β-actin: Sense: 5'-AGA TCC ACA ACG GAT ACA TT-3'; Antisense: 5'-TCC CTC AAG ATT GTC AGC AA-3', amplified cDNA length: 309 bp.

Statistical analysis

The data were representative of a normal distribution and accordingly the results were expressed as mean ± SE. The stress-strain curve for each direction was fitted using the exponential function equation $S = (S^* + \beta)\, e^{\alpha (E-E^*)} - \beta$.S* and E* are the stress and strain at a physiological reference level[5]. The constants α and β from the above exponential function were used for the statistical evaluation of the stress-strain data. Analysis of variance was used to detect differences in the parameters and groups (Sigmastat 2.0™). The results were regarded as significant when $P < 0.05$.

RESULTS

Blood glucose and serum insulin levels

Blood glucose and serum insulin levels at the end of the experiment are shown in Figure 2. The blood glucose level was 4-fold higher in the DM group compared with the control group (Figure 2A, $P < 0.01$). The serum insulin level was significantly lower in the DM group compared with the control group (Figure 2B, $P < 0.01$). Compared with the DM group, the blood glucose and insulin levels did not significantly change in the T1 and T2 groups.

The weight/cm, wall thickness and wall area

The wet weight per unit length (Figure 3A), no-load wall thickness (Figure 3B) and cross-sectional wall area (Figure 3C) of the esophageal and intestinal segments increased in the DM group compared with the control group ($P < 0.01$, $P < 0.05$). After treatment with T1, the wall thickness decreased in all segments (Figure 3B, $P < 0.05$), whereas the wet weight and wall area decreased in the duodenal, jejunal and ileal segments (Figure 3A, D, $P < 0.01$, $P < 0.05$), but not in the esophageal segment ($P = 0.12$). All the above parameters did not change significantly in the T2 group.

Opening angle and residual strain

At the end of the experiment, the opening angle of all three intestinal segments was significantly increased in

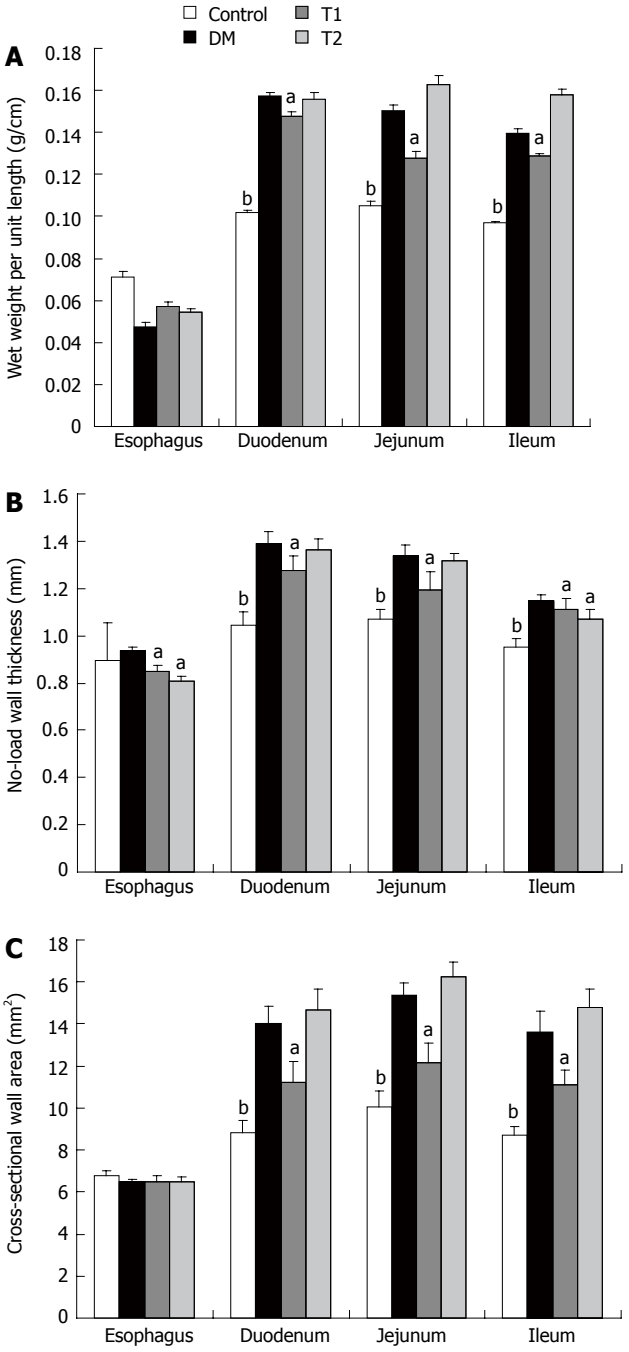


Figure 3 Wet weight per unit length, wall thickness and wall area in different segments of the different groups. A: Wet weight per unit length; B: Wall thickness; C: Wall area. [a]*P* < 0.05, [b]*P* < 0.01 *vs* DM group. DM: Diabetic control; T1: High dose; T2: Low dose.

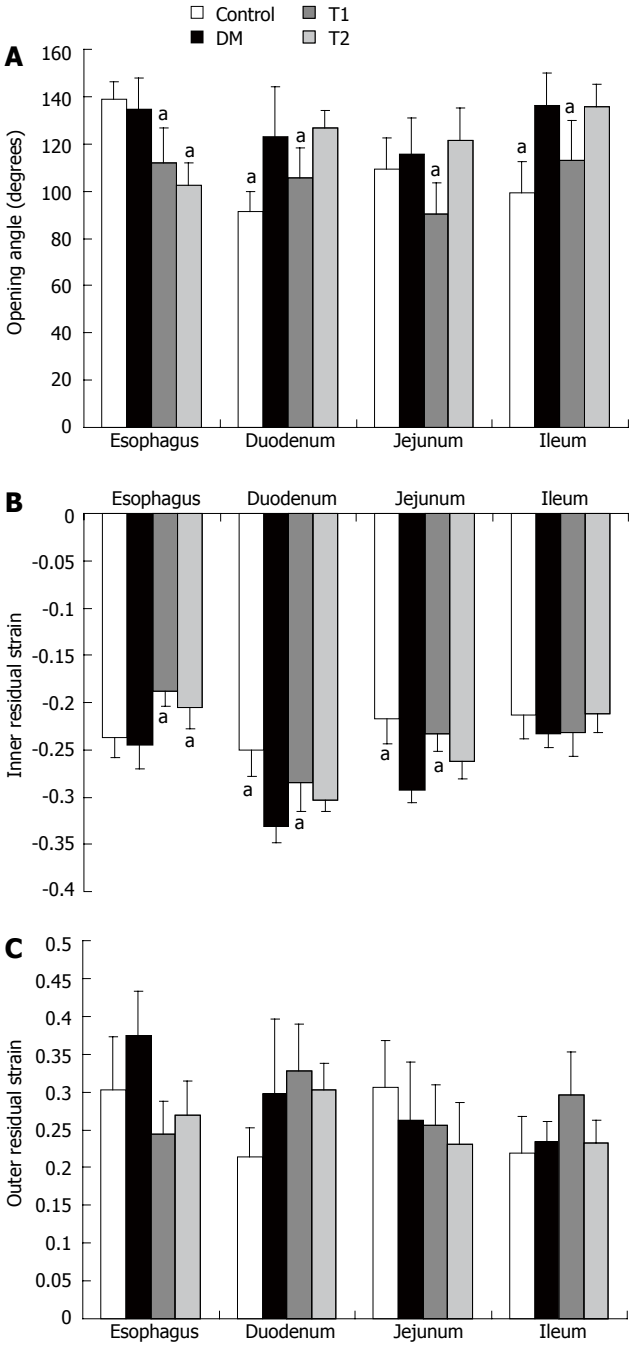


Figure 4 Opening angle, inner residual strain and outer residual strain in different segments of the groups. A: Opening angle; B: Inner residual strain; C: Outer residual strain. [a]*P* < 0.05 *vs* DM group. DM: Diabetic control; T1: High dose; T2: Low dose.

the DM group compared with the control group (Figure 4A, $P < 0.05$). However, the opening angle in the esophageal segment did not change. Treatment with high dose TWAJJ decreased the opening angle in all segments studied (Figure 4A, $P < 0.05$). Interestingly, the opening angle of the esophageal segment was also decreased in the T2 group ($P < 0.05$).

A similar pattern to that found in the opening angle was found in the inner residual strain for all segments and groups, with the exception of the ileal segment (Figure 4B), where no difference was found between the different groups ($P > 0.05$). The outer residual strain did not differ between the groups and segments (Figure 4C).

Stress-strain distribution

At the end of the experiment, the stress-strain analysis showed that the circumferential stress-strain curves of esophageal and jejunal segments (Figure 5A, C), and the longitudinal stress-strain curve of the esophageal segment (Figure 5B) in the DM group shifted to the left

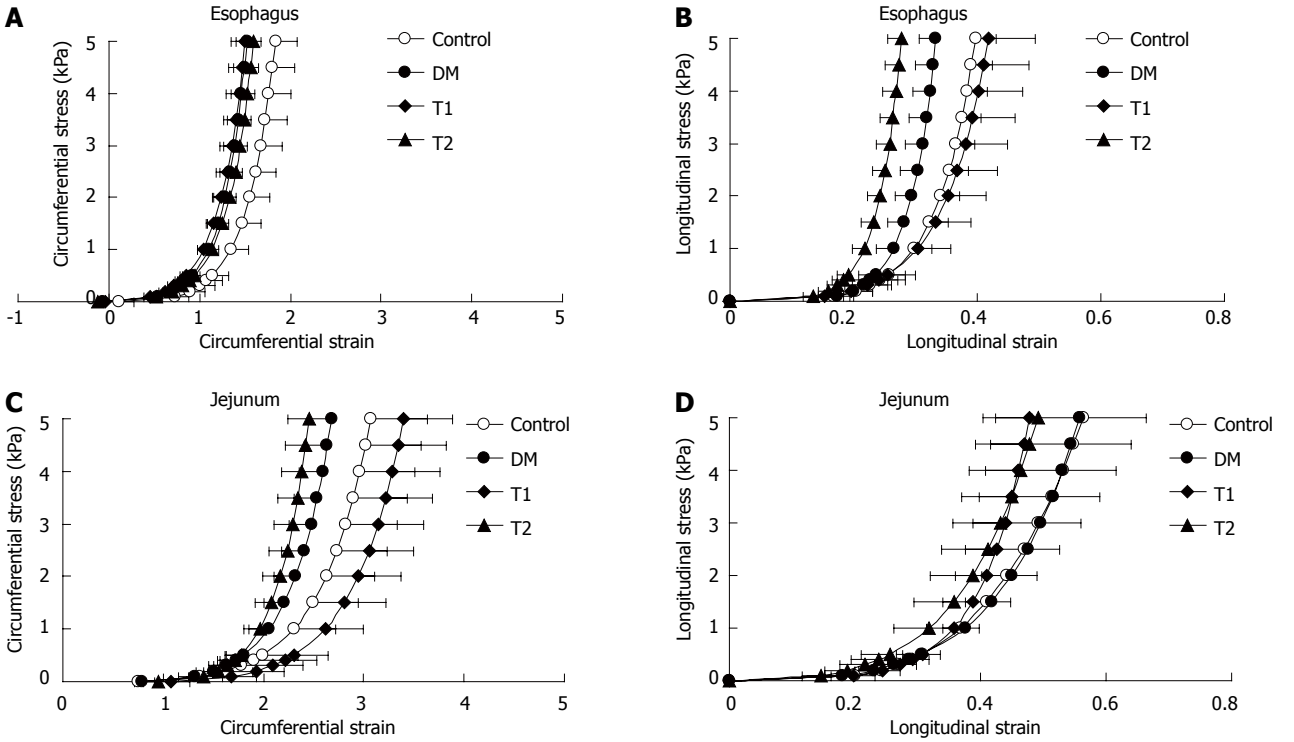


Figure 5 Circumferential and longitudinal stress-strain curves of esophageal and jejunal segments in the different groups. DM: Diabetic control; T1: High dose; T2: Low dose.

Table 1 Comparison of constant a among the different groups

	Circumferential direction		Longitudinal direction	
	Esophagus	Jejunum	Esophagus	Jejunum
Control (n = 10)	3.56 ± 0.35	2.65 ± 0.37	18.33 ± 2.36	13.51 ± 3.66
DM (n = 9)	4.59 ± 0.79[a]	3.81 ± 0.31[a]	27.62 ± 3.93[a]	14.79 ± 2.26
T1 (n = 9)	3.77 ± 0.39	2.55 ± 0.51[c]	19.43 ± 4.13[c]	14.67 ± 1.56
T2 (n = 9)	3.84 ± 0.44	4.02 ± 0.71[a]	27.82 ± 1.74[a]	15.41 ± 4.36

[a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs* DM group. DM: Diabetic control; T1: High dose; T2: Low dose.

compared with those in the control group, indicating that the diabetic esophageal and intestinal wall became stiffer. Calculation of the mechanical constants demonstrated a difference between the DM group and the control group ($P < 0.05$, Table 1). However, the longitudinal stress-strain distribution of the jejunal segment did not differ between the DM and control groups ($P > 0.05$, Figure 5D). High dose TWAJJ (T1) decreased the stiffness of the esophageal wall in the longitudinal direction (Figure 5B and Table 1, $P < 0.05$) and the intestinal wall in the circumferential direction (Figure 5C and Table 1, $P < 0.05$). Low dose TWAJJ did not improve the stiffening of the esophageal and jejunal wall caused by diabetes ($P > 0.05$, Figure 5 and Table 1).

Receptor of advanced glycation end-products mRNA level

RAGE mRNA level of the jejunal segment increased in the DM group compared to the control group (Figure 6, $P < 0.01$). T1 treatment reduced the RAGE mRNA level (Figure 6, $P < 0.01$). We did not observe a significant difference between the T2 group and the DM group (Figure 6, $P > 0.05$).

DISCUSSION

Diabetes mellitus is a pandemic disease. According to WHO´s statistical report; 370 million diabetic patients are expected by 2030. The serious impact caused by diabetes and its complications does not only affect patients but is also a heavy financial burden to the health care system and on families[20]. DGID is a common complication of diabetes. A previous study showed that prominent proliferations of the GI wall, especially the small intestine and esophagus mucosae, occur in experimental diabetes[21]. Other studies demonstrated morphological and biomechanical remodeling of the upper GI tract in diabetes[9-10,22], which may be caused by over-expression of AGE and RAGE in the diabetic GI tract. Furthermore, a linear association between the glucose level and morphometric and biomechanical remodeling was observed[23].

In the view of traditional Chinese medicine, DGID is in the category of fullness, epigastric pain, vomiting, diarrhea, and constipation concurrent with diabetes. The major syndrome of DGID includes spleen deficiency and qi stagnation. Therefore, the principal treatment is to strengthen the spleen and remove stagnation. TWAJJ is a practical Chinese medicinal compound for DGID[24-26] and is composed of several herbs. Atractylodes mac-

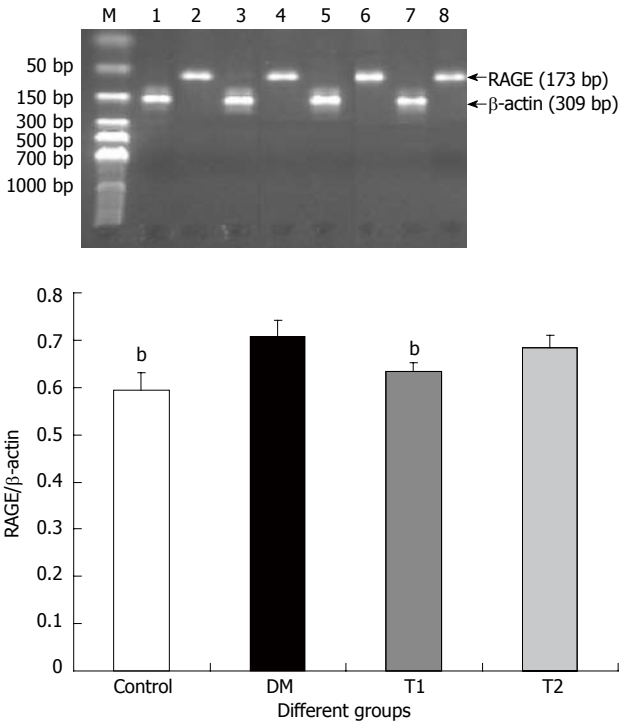


Figure 6 Receptor of advanced glycation end-products mRNA level of the jejunal segment in the different groups. $^{b}P < 0.01$ *vs* DM group. M: Ladder marker; 1, 3, 5, 7: β-actin; 2, 4, 6, 8: RAGE; 1, 2: Control; 3, 4: DM; 5, 6: T1; 7, 8: T2. DM: Diabetic control; T1: High dose; T2: Low dose; RAGE: Receptor of advanced glycation end-products.

rocephala can strengthen the spleen, harmonize the stomach, regulate GI motility and protect the gastric mucosa[27]. Citrus aurantium and wine rhubarb move qi and accumulation. Pinellia downbears counterflow and inhibits vomiting. Ginseng tones qi and has a beneficial affect on body fluid. Citrus aurantium has a non-competitive antagonist effect on acetylcholine and histamine in intestinal smooth muscle contraction, possibly by influencing Ca^{2+} channels. Its total alkaloid level can prevent vomiting and can promote GI tract movement in rats[28-30]. The active ingredients of ginseng ginsenosides can reduce blood sugar by stimulating insulin secretion and anti-apoptosis of pancreatic β-cells[31,32]. Furthermore, the results of a retrospective clinical study on TWAJJ treatment in DGID patients have been reported[24,25]. After treatment with TWAJJ, symptoms such as fullness, eructation, nausea (vomiting) and constipation were significantly improved. Although the fasting blood glucose tended to decrease after treatment, this decrease was not statistically significant. However, clarification of the clinical validity of TWAJJ in randomized controlled clinical trials is necessary. In this study, we also found that TWAJJ had a tendency to decrease blood glucose and increase serum insulin, however, statistical significance was not reached. Therefore, we consider that the functional mechanism of TWAJJ in DGID is through other pathways rather than through blood glucose regulation.

The esophagus and small intestine are tubular organs. One important function is transportation of food by wall movement; therefore the biomechanical properties of the wall are important for this function[8]. The biomechanical properties of the esophagus and small intestine depend on their structure and can be evaluated by the opening angle, residual stress and strain, and stress-strain relationship[11]. Previous studies have demonstrated that the esophageal wall[12,33,34] and intestinal wall[9-11] were remodeled during the development of diabetes. Therefore, it is important to determine whether TWAJJ can improve DGID through a pathway involved in changing the biomechanical properties of the GI tract.

The present study confirmed previous findings that morphometric and biomechanical remodeling of the esophageal and intestinal wall occur in STZ-induced diabetic rats. Although the treatment with TWAJJ did not significantly change the blood glucose and serum insulin levels, high dose TWAJJ, to a large extent, improved the morphometric and biomechanical remodeling caused by diabetes. The improvement in morphometric remodeling was expressed as reduced wall thickness and area. Improvements in biomechanical remodeling were expressed as reduced opening angle, reduced absolute value of residual strain and decreased wall stiffness. The present study indicated that the effect of TWAJJ on improvements in DGID in the clinic may be partially associated with improvements in biomechanical remodeling of the GI wall. Due to the complex geometry of the stomach[22,35], we did not focus on the biomechanical parameters of the gastric wall in the present study. Future studies may focus on the effect of TWAJJ on the stomach due to the importance of stomach function in diabetic patients.

The alterations in residual strain and wall stiffness in diabetes will change the tension and stress distribution in the location of mechanosensitive afferents in the GI tract[36,37]. Therefore, biomechanical remodeling of the diabetic GI wall indirectly affects GI motor function. High dose TWAJJ partially restored the changes in biomechanical properties caused by diabetes. This may be associated with improved motor and sensory function of the diabetic GI tract.

AGEs is a heterogeneous group of macromolecules formed by the non-enzymatic glycation of proteins, lipids and nucleic acids[38]. Endogenous AGEs are generated at higher rates in diabetes due to abnormal glucose metabolism. Chronic high blood glucose can cause excessive glycoprotein accumulation in the body including the GI tract causing GI wall stiffening[8]. There is growing evidence to show that AGEs and RAGE are implicated in various disorders. AGE levels in serum and tissues are associated with chronic complications of DM including DGID. Furthermore, RAGE is involved in signal transduction of AGEs in a variety of cells[39]. Our previous studies showed that AGE and RAGE were over-expressed in diabetic GI tissues[40]. In the present study, we found that the RAGE mRNA level in the jejunal segment increased in diabetic rats compared with normal rats. Following high dose TWAJJ administration, this alternation was reversed to the normal state. Therefore, the observed

improvement in the mechanical factors due to TWAJJ treatment in DGID may be through the inhibition of AGEs accumulation. However, in future studies we need to clarify this mechanism by immunohistochemistry of RAGE to demonstrate whether the change in mRNA expression actually translates to protein expression. Furthermore, it is also important to study whether specific knockdown of RAGE in the GI mucosa can improve biomechanical remodeling of the GI tract in diabetes and reverse DGID.

In conclusion, high dose TWAJJ treatment improves biomechanical and morphometric remodeling of the diabetic esophageal and intestinal wall. The mechanism may, at least partly, be explained by decreased RAGE mRNA levels in STZ-induced diabetic rats. Therefore, it seems feasible to develop Chinese herbs, such as TWAJJ, to improve the morphometric and biomechanical remodeling caused by diabetes. This may have an impact on GI dysfunction in diabetes and be used in clinical practice.

COMMENTS

Background

Diabetic gastrointestinal disorder (DGID) is a common complication of diabetes. However, neither the pathophysiology nor the pathogenesis of patients' gastrointestinal (GI) symptoms associated with diabetes are well understood. Furthermore, many patients with DGID do not receive appropriate treatment. Although Western medicine can partially improve the clinical symptoms, it does not fundamentally reverse the diabetes-induced changes and results in a very high relapse rate. In the clinic, it was shown that Tangweian Jianji (TWAJJ) significantly improved DGID with a low relapse rate, however, the mechanism involved in this improvement is not fully understood.

Research frontiers

The GI tract is functionally subjected to dimensional changes. Hence, biomechanical properties such as the stress-strain relationship are of particular importance. Several studies have demonstrated that diabetes induces GI morphological and biomechanical remodeling. The non-enzymatic glycation of the intestinal wall in diabetes is associated with remodeling. Therefore, the present study investigated whether TWAJJ treatment can improve morphometric and biomechanical remodeling of the GI tract in streptozotocin (STZ)-induced diabetic rats. Furthermore, the receptor of advanced glycation end-products (RAGE) mRNA level in the jejunal tissues was also investigated to explore the possible mechanism of TWAJJ in the treatment of DGID.

Innovations and breakthroughs

Compared with previous studies, this study is the first to use biomechanical test methods combined with RAGE detection to investigate the mechanism of the effect of the Chinese medicine, TWAJJ, on diabetic GI dysfunction. We showed that high dose TWAJJ improves the biomechanical and morphometric remodeling of the diabetic esophageal and intestinal walls. The mechanism may, at least partly, be related to the decreased RAGE mRNA levels in STZ-induced diabetic rats. As knowledge of GI biomechanics is new and was only recently developed and the non-enzymatic glycation of the GI tract is closely related to its biomechanical properties, determination of biomechanical properties and expression of advanced glycation end products and their receptor (AGE/RAGE) may be useful tools to investigate the mechanism of TWAJJ in DGID.

Applications

The findings of this study suggest that it seems feasible to develop Chinese herbs, such as TWAJJ, to improve the morphometric and biomechanical remodeling caused by diabetes. This may have an impact on GI dysfunction in diabetes and be used in clinical practice.

Terminology

DGID is a common complication of diabetes related to the GI tract. Up to 76% of diabetic patients express GI symptoms including dysphagia, early satiety, reflux, constipation, abdominal pain, nausea, vomiting and diarrhea; GI biomechanical properties are used to define the GI wall deformation (strain) and force (stress), which can be described by the opening angle, residual stress and strain, stress-strain relationship, stress relaxation and creep; TWAJJ is a practical Chinese medicinal compound for diabetic gastrointestinal dysfunction and is composed of several herbs including Citrus aurantium, Codonopsis, fried Atractylodes and wine Rhubarb.

Peer review

This is an interesting article. DGID is clearly a big topic especially in developed countries, thus this topic is interesting. Further, it was also fascinating to see that high dose TWAJJ was able to "treat" DGID without treating hyperglycemia. The manuscript is well written, the experiments are well planned, methods are adequately described and suitable to achieve the goals and results are appropriately described.

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BRIEF ARTICLE

Clinical usefulness of adherence to gastro-esophageal reflux disease guideline by Spanish gastroenterologists

Fermín Mearin, Julio Ponce, Marta Ponce, Agustín Balboa, Miguel A González, Javier Zapardiel

Fermín Mearin, Agustín Balboa, Service of Gastroenterology, Centro Medico Teknon, 08022 Barcelona, Spain
Julio Ponce, Digestive Disease Unit, Hospital Quiron, 46010 Valencia, Spain
Julio Ponce, Marta Ponce, Centro de Investigacion Biomedica en Red: Enfermedades Hepaticasy Digestivas, 08036 Barcelona, Spain
Marta Ponce, Service of Gastroenterology, Hospital Universitario La Fe, 46026 Valencia, Spain
Miguel A González, Biostatistics, Innovex-Grupo Quintiles Espana, 28033 Madrid, Spain
Javier Zapardiel, Medical Department AstraZeneca Farmaceutica, 28033 Madrid, Spain
Author contributions: Mearin F studied concept and design, analysed and interpreted the data, drafted the manuscript; Ponce J studied concept and design, analysed and interpreted the data, drafted the manuscript; Ponce M drafted the manuscript; Balboa A analysed and interpreted the data, drafted the manuscript; González MA acquired the data and statistical analysis; Zapardiel J analysed and interpreted the data, drafted the manuscript.
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Correspondence to: Fermín Mearin, MD, PhD, Service of Gastroenterology, Centro Medico Teknon, C/Vilana 12, 08022 Barcelona, Spain. fmearinm@meditex.es
Telephone: +34-93-3933143 Fax: +34-93-3936032
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Abstract

AIM: To investigate usefulness of adherence to gastro-esophageal reflux disease (GERD) guideline established by the Spanish Association of Gastroenterology.

METHODS: Prospective, observational and multicentre study of 301 patients with typical symptoms of GERD who should be managed in accordance with guidelines and were attended by gastroenterologists in daily practice. Patients (aged > 18 years) were eligible for inclusion if they had typical symptoms of GERD (heartburn and/or acid regurgitation) as the major complaint in the presence or absence of accompanying atypical symptoms, such as dyspeptic symptoms and/or supraesophageal symptoms. Diagnostic and therapeutic decisions should be made based on specific recommendations of the Spanish clinical practice guideline for GERD which is a widely disseminated and well known instrument among Spanish in digestive disease specialists.

RESULTS: Endoscopy was indicated in 123 (41%) patients: 50 with alarm symptoms, 32 with age > 50 years without alarm symptom. Seventy-two patients (58.5%) had esophagitis (grade A, 23, grade B, 28, grade C, 18, grade D, 3). In the presence of alarm symptoms, endoscopy was indicated consistently with recommendations in 98% of cases. However, in the absence of alarm symptoms, endoscopy was indicated in 33% of patients > 50 years (not recommended by the guideline). Adherence for proton pump inhibitors (PPIs) therapy was 80%, but doses prescribed were lower (half) in 5% of cases and higher (double) in 15%. Adherence regarding duration of PPI therapy was 69%; duration was shorter than recommended in 1% (4 wk in esophagitis grades C-D) or longer in 30% (8 wk in esophagitis grades A-B or in patients without endoscopy). Treatment response was higher when PPI doses were consistent with guidelines, although differences were not significant (95% *vs* 85%).

CONCLUSION: GERD guideline compliance was quite good although endoscopy was over indicated in patients > 50 years without alarm symptoms; PPIs were prescribed at higher doses and longer duration.

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Key words: Gastro-esophageal reflux disease; Clinical practice guidelines; Adherence to guidelines; Treatment of gastro-esophageal reflux disease

Peer reviewer: Jose Liberato Ferreira Caboclo, Professor, Department of Surgery, Institution of FAMERP, Rua Antônio de Godoy, 4120 São José do Rio Preto, Brazil

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INTRODUCTION

Gastro-esophageal reflux disease (GERD) is one of the most prevalent gastrointestinal disorders in the general population[1]. GERD diagnosis is made mainly according to the presence of symptoms. Patients with heartburn and/or regurgitation (typical GERD symptoms) received the diagnosis. Typical symptoms of GERD are experienced by 25% of people at least once a month, 12% at least once per week, and 5% describe daily symptoms[2]. The prevalence of GERD among the Spanish population is 15%, with 33% and 22% of subjects reporting monthly episodes of heartburn and regurgitation, respectively[3]. Reported consultation rates range from 5% to 56%[4].

GERD is perceived as a benign disease but the spectrum of chronicity and severity of upper gastrointestinal symptoms frequently affects health-related quality of life (HRQoL)[5]. The wide-ranging effects of GERD on health and well-being can have consequences for the work productivity, psychological and social performance of affected individuals, particularly in patients with severe symptoms or night time acid reflux and sleep disturbance[5-8]. In some respects the HRQoL burden of these patients is similar to or greater than that observed in patients with conditions such as diabetes, hypertension or coronary heart disease[9,10].

Although treatment with proton pump inhibitors (PPIs) is satisfactory in many cases and results in cicatrisation of esophagitis when esophagitis is present, complete disappearance of symptoms is difficult to achieve[11]. Nevertheless, in many cases PPI therapy leads to rapid symptom improvement that is associated with a better HRQoL. For example, in a large Italian study, 96% of patients were satisfied with the results of 4 wk of PPI therapy and the average HRQoL score improved significantly during this period[12]. An effective management strategy is particularly important because, as mentioned, GERD imposes a significant burden of illness[13,14]. For that purpose several clinical practice guidelines have been developed by different medical societies and expert consensus meetings[15-19]. However, although many national and international guidelines are currently available, the impact of these recommendations on the behaviour of physicians has generally been limited[20-25]. Only in some disease states guidelines have been shown to influence physician behaviour, which depends on the prevalence of the disorder and access to the guidelines among other factors[26-28].

The objective of this study was to assess the level of adherence of gastroenterologists to the Spanish clinical guidelines for GERD and the results obtained in the care of patients with typical symptoms of GERD attended in daily practice.

MATERIALS AND METHODS

Study design

This multicentre and observational study was prospectively designed with two purposes: (1) to investigate whether patients with typical GERD symptoms seeking consultation from a specialist in gastroenterology were managed in accordance with the clinical practice guideline for GERD developed by the Spanish Association of Gastroenterology, the Spanish Society of Family and Community Medicine and the Iberoamerican Cochrane Center[15]; and (2) to assess the results obtained. The study was focused on the "acute phase" of GERD (4-8 wk after diagnosis) and not on the long-term; therefore, evaluation of maintenance treatment was not done. Thirty-three gastroenterologist from 33 different medical centers throughout Spain participated in the study, that was conducted under routine clinical practice conditions. Gastroenterologists participating in the study were not aware they were included in an adherence study. Consecutive patients were recruited between December 12, 2007 and November 5, 2008. All patients provided written informed consent. The protocol was approved by the Ethics Committees of the participating centers.

Inclusion and exclusion criteria

Consecutive patients (aged > 18 years) attending to gastroenterologists offices were eligible for inclusion if they had typical symptoms of GERD (heartburn and/or acid regurgitation) more than twice a week for at least 2 mo as the major complaint, in the presence or absence of accompanying atypical symptoms, such as dyspeptic symptoms and/or supraesophageal symptoms. All enrolled patients were required to sign the informed consent. Patients who had taken drug treatment for GERD within 2 mo prior to the study were excluded. Also, patients were not eligible they were currently taking non-steroidal anti-inflammatory drugs or platelet antiaggregant agents as well as if previous treatment with PPIs had been unsuccessful. Patients with history of esophageal or gastric surgery except for suture repair of a perforated gastric ulcer were excluded.

Study procedures

The study protocol included a baseline visit and a final visit, which was scheduled after 4 wk or 8 wk. At the baseline visit, eligibility criteria were assessed and the following data were recorded: demographic and anthropometric variables, and GERD-related symptoms including

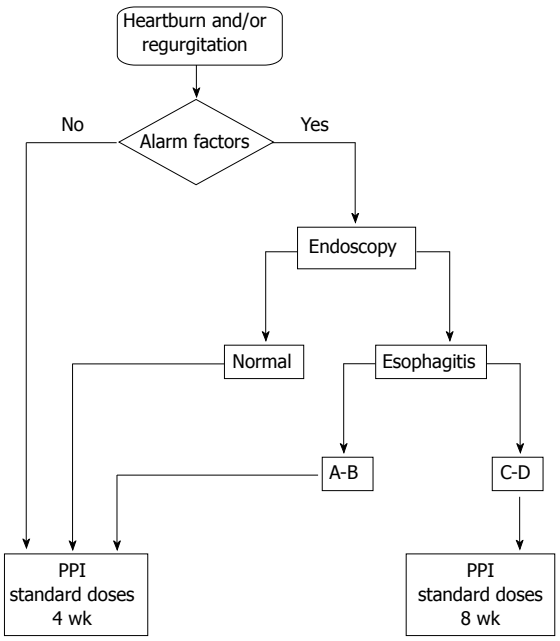


Figure 1 Management algorithm of patients with heartburn or regurgitation that should be used according to recommendations of the Spanish Clinical Practice Guideline. PPI: Proton pump inhibitor.

duration of symptoms, previous drug treatment, and frequency and intensity of typical symptoms. The frequency of each symptom was graded as 0 = absent or present less than 2 d per month, 1 = present more than 2 d per month, 2 = present weekly less than 3 d, and 3 = present 3 or more days per week. Intensity was graded as 0 = none, 1 = awareness of symptom but easily tolerated, 2 = discomfort sufficient to cause interference with normal activities, and 3 = incapacitating, with inability to perform normal activities. The product of frequency and intensity was used to define the severity of each symptom, which was expressed as the absolute number (0-1 = absent or irrelevant, 2 = mild, 3-4 = moderate, 6 = severe, and 9 = very severe).

Diagnostic and therapeutic decisions should be made based on specific recommendations of the Spanish clinical practice guideline for GERD[15], which is a widely disseminated and apparently well known instrument among specialists in digestive disease. The algorithm for the diagnosis and treatment of patients with GERD is summarized in the Figure 1. When upper gastrointestinal endoscopy was indicated, reasons and endoscopic findings should be recorded. The presence and severity of erosive esophagitis were determined using the Los Angeles classification[29]. According to the guideline, in the absence of erosive esophagitis or mild esophagitis (Los Angeles grade A or B) the duration of treatment was 4 wk but in the presence of esophagitis grades C and D, the duration of treatment was 8 wk. Treatment-related data included prescription of PPI, dose and duration of treatment (4 wk or 8 wk), and non-pharmacological measures (acid reflux diet, elevated position of the bed). The PPI selection (omeprazole, lansoprazole, pantoprazole, rabeprazole or esomeprazole) was made by individual medical preference.

Assessments at the end of treatment (4 wk or 8 wk) included frequency and intensity of symptoms to quantify the severity of disease after PPI treatment. Response to treatment was considered when patients became asymptomatic, that is, in the category of "absent or irrelevant". Patients were interviewed regarding their compliance with treatment, which was classified as excellent (> 95%), very good (> 90%), good (> 80%), fair (≥ 70%), bad (< 70%) and very bad (< 50%).

Statistical analysis

Sample size calculation was based on proportion of symptomatic treatment response after 4-8 wk. Considering a treatment response around 58%, with a ± 6% deviation, the needed sample size was 260 patients. Expecting a 15% lost during follow-up the final calculated sample size was 306 patients.

Categorical data are expressed as absolute number and percentages, and continuous data as mean and standard deviation. Response to PPI therapy according to adherence to guidelines in the doses prescribed was analyzed with the χ^2 test or fisher exact test. All analyses were performed using SAS 8.2 (SAS Institute, Cary, NC). A P value of less than 0.05 was considered statistically significant.

RESULTS

A total of 306 patients were recruited and 301 patients (58.5% men; age of 45 ± 14 years) fulfilled the inclusion criteria and were included in the study. All of them completed follow-up. The remaining 5 patients were excluded because of refusal to take part in the study (n = 2) and lack of fulfilment of the inclusion/exclusion criteria (n = 3). Mean time from the onset of GERD symptoms was 4.5 ± 6.3 years. Previous pharmacological treatment for GERD (not within 2 mo prior to the study) was recorded in 156 patients (51.9%), 87 of which had been treated with PPIs. Presenting complaints were heartburn in 99% of cases (nocturnal heartburn in 78%), regurgitation in 86%, and both heartburn and regurgitation in 85%. Only 3 patients complained of regurgitation only. A total of 273 patients (90.7%) presented dyspeptic symptoms and 174 (57.8%) supraesophageal symptoms. Distribution of heartburn, nocturnal heartburn and regurgitation according to severity of symptoms is shown in Figure 2. Symptoms were rated as severe or very severe in 56% of cases for heartburn, 34% for nocturnal heartburn and 35% for regurgitation.

Upper gastrointestinal endoscopy was performed in 123 patients (40.8%). According to participating gastroenterologists opinion indication of endoscopy was justified because of alarm symptoms in 50 patients and age over 50 years in 32. Adherence to the guidelines in relation to indication of upper gastrointestinal endoscopy

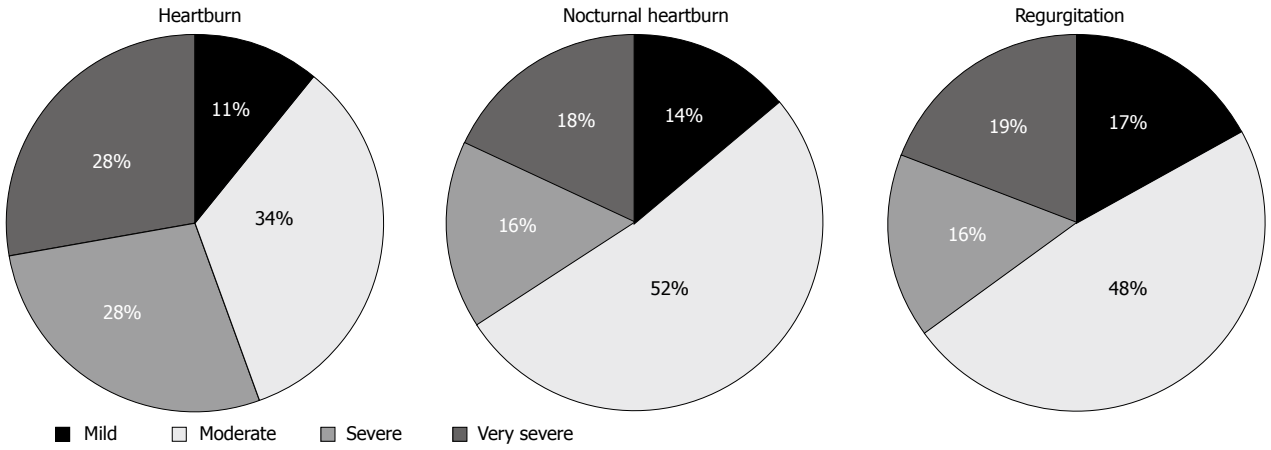


Figure 2 Distribution of typical gastro-esophageal reflux disease symptoms according to severity.

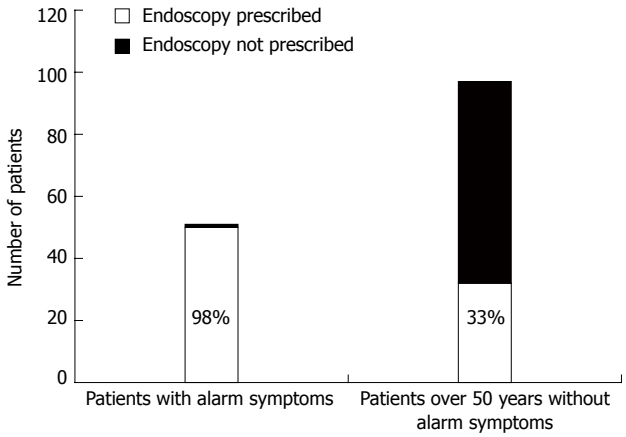


Figure 3 Upper gastrointestinal endoscopy was prescribed in 98% of patients with alarm symptoms (in accordance with the guideline) and in 33% of patients older than 50 years of age without alarm symptoms (not in accordance with the guideline).

was good due to the presence of alarm symptoms in 98% of cases. On the other hand, endoscopy was performed in 32 of 97 patients (33%) older than 50 years of age without alarm symptoms (Figure 3).

Esophagitis was diagnosed in 72 (58.3%) patients (grade A, 23, grade B, 28, grade C, 18, grade D, 3). Metaplastic changes of the esophageal mucosa suggestive of Barrett's oesophagus were documented in 9 patients and peptic stenosis in 1. Hiatal hernia was reported in 37 patients and Schatzki ring in 5.

Treatment with PPIs was prescribed to 298 patients (99%). In most cases (80%), there was adherence to the guidelines as the recommended dose of PPI was prescribed. However, doses prescribed were lower (half) in 4.8% of cases and higher (double) in 14.7%. The duration of PPI therapy was 4 wk in 63% of cases and 8 wk in 37%. In respect to duration of PPI therapy, adherence to guidelines was shown in 69% of cases (62% for the indication of 4 wk and 7% for 8 wk). Lack of adherence (31% of cases) included duration of treatment of 4 wk in patients with esophagitis grades C-D (0.7%) or 8 wk in patients esophagitis grades A-B (7.8%) or in patients without endoscopic studies (22.8%).

Compliance with pharmacological treatment was excellent in 58% of cases, very good in 30%, good in 8%, fair in 3% and bad or very bad in 1%. Acid reflux disease diet was recommended in 79% of patients and raising the bed position in 34%.

Response to PPI therapy was higher among patients who received PPI doses recommended by the guidelines as compared with patients treated with lower doses (95% *vs* 85%), although differences were not statistically significant ($P = 0.2$). Treatment response was similar in patients in which adherence to guidelines regarding duration of treatment was good or poor (95% *vs* 93%; $P = 0.5$).

Symptomatic response to treatment according to esophagitis grade was 87% in grade A, 89% in grade B and 100% in grades C and D. The indication to perform an upper gastrointestinal endoscopy had no effect on the rate of response to PPI treatment ($P = 0.9$) or whether there was good or poor adherence to the guidelines in terms of indication of the endoscopic study ($P = 0.7$).

DISCUSSION

GERD is a chronic condition in many cases and frequently requires prolonged therapy. Although complications are infrequent, the symptoms of reflux disease have a profound effect on quality of life and work performance, making GERD an expensive disease to manage. When different management strategies are compared, the cost of each strategy must be balanced against its effectiveness. For that purpose, various clinical practice guidelines have been developed based on the scientific evidence and recommendations made by experts[15-19]. In 2007, the Spanish Association of Gastroenterology, the Spanish Society of Family and Community Medicine and the Iberoamerican Cochrane Center developed a clinical practice guideline for GERD, in which two of the authors have collaborated (FM, JP), which was distributed throughout Spain both in printed version and through

the Internet[15]. Afterwards, it was investigated whether the GERD guideline had a practical impact through the Spanish gastroenterologists in the care of patients with GERD in routine daily practice.

The main findings of this study can be summarized as follows: (1) upper gastrointestinal endoscopy was requested in 41% of patients, mainly because of the presence of alarm symptoms but also because of age over 50 years without alarm symptoms; (2) severe esophagitis was infrequently diagnosed even though patients were selected for endoscopy; (3) in almost all cases (99%), treatment with PPIs was prescribed, in the majority of cases (80%) in accordance with doses recommended in the guidelines (lower doses were prescribed in only 5% of cases and higher doses in 15%); (4) adherence regarding duration of PPIs therapy was lower (69%) and most non-compliant behaviours were related to prolonged duration of treatment in patients with mild esophagitis or in those in which endoscopy was not performed; and (5) response to PPI treatment was higher in patients treated with doses of PPIs recommended in the clinical practice guidelines as compared with patients receiving other doses, although the difference was not statistically significant (95% *vs* 85%).

It is to note that, although GERD guideline compliance was quite good, endoscopy was overindicated. Thus, it was prescribed in 32 out of 97 patients (33%) older than 50 years of age without alarm symptoms. We have to emphasize here that not in the Spanish guideline neither in other very prestigious ones[30,31] is endoscopy recommended in patients with typical GERD symptoms (heartburn and/or regurgitation) older than 50 years and no alarm symptoms.

GERD is a very frequent cause of consultation in specialized gastroenterology practices as well as in the primary care setting. Consultation for GERD is associated with increased symptom severity and frequency, interference with social activities, sleep disturbance, higher levels of comorbidity, and psychological distress. Patients are less likely to consult if they feel that their doctor would trivialise their symptoms[4]. GERD has a negative impact on HRQoL being involved factors such as female gender, increased body mass index and nocturnal symptoms[5]. Nevertheless, when symptoms are properly treated, HRQoL improves in most of the cases. Thus, in a 5-year follow-up study of a large GERD population under routine care only a small minority of patients reported a clinically relevant decrease in HRQoL[32].

Although several clinical practice guidelines for GERD have been developed and published, to our knowledge, their clinical impact on gastroenterologists practice has not been evaluated. On the other hand, it is known that current GERD guidelines are infrequently used by primary care physicians. It has been shown that among 352 practitioners from 17 countries only 33% used an international and 14% used a national guideline in managing GERD patients[33]. However, in a retrospective Australian study it was shown a significant improvements in the diagnosis and management of GERD after a clinical guideline distribution and a 3-year self-audit process revealed a decrease in use of endoscopy, improved identification of risk factors, increase in recommendations for patient weight loss and reduction in use of medications that may exacerbate reflux symptoms[34]. Regarding antisecretory therapy it is well accepted that these medication are frequently overprescribed; when concordance between use of PPIs and prescribing guidelines was evaluated drug utilisation data indicated widespread use of PPIs outside current prescribing guidelines[35]. Another study tried to evaluate variability in dyspepsia management among Italian general practitioners; it was found that 44% of endoscopies prescribed for uninvestigated patients (more than half complaining of GERD symptoms) did not comply with the European Society for Primary Care Gastroenterology guideline[36].

In conclusion, compliance with the clinical practice guidelines for GERD by Spanish gastroenterologists is quite good, although a trend to indicate endoscopy in patients older than 50 years without alarm symptoms and treatments with higher PPI doses and longer duration than recommended was observed. Diagnosis of severe esophagitis is extremely infrequent, even in selected patients for endoscopy. Therapeutic response trended to be better when guideline recommendations were followed.

COMMENTS

Background

Gastro-esophageal reflux disease (GERD) is one of the most prevalent gastrointestinal disorders in the general population. GERD diagnosis is made mainly according to the presence of symptoms, especially heartburn and/or regurgitation. An effective management strategy is particularly important because GERD imposes a significant burden of illness. For that purpose several clinical practice guidelines have been developed by different medical societies and expert consensus meetings. However, the impact of these recommendations on the behaviour of physicians has generally been limited.

Research frontiers

It is a prospective, observational and multicentre study evaluating 301 patients with typical symptoms of GERD who should be managed in accordance with guidelines and were attended by gastroenterologists in daily practice. Diagnostic and therapeutic decisions should be made based on specific recommendations of the Spanish clinical practice guideline for GERD which is a widely disseminated and well known instrument among Spanish in digestive disease specialists.

Innovations and breakthroughs

The main findings of this study can be summarized as follows: Upper gastrointestinal endoscopy was requested in 41% of patients, mainly because of the presence of alarm symptoms but also because of age over 50 years without alarm symptoms. Severe esophagitis was infrequently diagnosed even though patients were selected for endoscopy.

Applications

In almost all cases (99%), treatment with Adherence for proton pump inhibitor (PPIs) was prescribed, in the majority of cases (80%) in accordance with doses recommended in the guidelines (lower doses were prescribed in only 5% of cases and higher doses in 15%). Adherence regarding duration of PPI therapy was lower (69%) and most non-compliant behaviours were related to prolonged duration of treatment in patients with mild esophagitis or in those in which endoscopy was not performed.

Terminology

Response to PPI treatment was higher in patients treated with doses of PPIs recommended in the clinical practice guidelines as compared with patients receiving other doses, although the difference was not statistically significant (95% *vs* 85%).

Peer review

Treatment practice guidelines are helpful in securing patients uniform treatment regardless of where they seek help. Such guidelines are of no use, however, if they are not followed. A study of adherence to guidelines is thus of significant importance. The presentation of the study is concise, and was approved by relevant ethics committees.

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BRIEF ARTICLE

IL28B polymorphism as a predictor of antiviral response in chronic hepatitis C

Andrzej Cieśla, Monika Bociąga-Jasik, Iwona Sobczyk-Krupiarz, Mikołaj K Głowacki, Danuta Owczarek, Dorota Cibor, Marek Sanak, Tomasz Mach

Andrzej Cieśla, Monika Bociąga-Jasik, Iwona Sobczyk-Krupiarz, Mikołaj K Głowacki, Danuta Owczarek, Dorota Cibor, Tomasz Mach, Department of Gastroenterology, Hepatology and Infectious Diseases, Jagiellonian University Medical College, 31-521 Kraków, Poland
Marek Sanak, Department of Molecular Biology and Clinical Genetics, II Department of Internal Medicine, Jagiellonian University Medical College, 31-066 Kraków, Poland
Author contributions: Cieśla A, Mach T, Sanak M designed the research; Cieśla A, Sobczyk-Krupiarz I, Głowacki MK, Cibor D performed the study; Sanak M performed the analytical part of study; Cieśla A, Mach T, Bociąga-Jasik M analyzed the data, Cieśla A, Mach T, Bociąga-Jasik M, Sobczyk-Krupiarz I, Głowacki MK, Owczarek D, Cibor D wrote the paper.
Correspondence to: Andrzej Cieśla, MD, Department of Gastroenterology, Hepatology and Infectious Diseases, Jagiellonian University Medical College, 31-521 Kraków, Poland. aciesla@su.krakow.pl
Telephone: +48-124-247340 Fax: +48-124-2473480
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Abstract

AIM: To evaluate the effect of single nucleotide polymorphisms of interleukin (IL)-28B, rs12979860 on progression and treatment response in chronic hepatitis C.

METHODS: Patients (n = 64; 37 men, 27 women; mean age, 44 ± 12 years) with chronic hepatitis C, genotype 1, received treatment with peg-interferon plus ribavirin. Genotyping of rs12979860 was performed on peripheral blood DNA. Histopathological assessment of necroinflammatory grade and fibrosis stage were scored using the METAVIR system on a liver biopsy sample before treatment. Serum viral load, aminotransferase activity, and insulin level were measured. Insulin resistance index, body mass index, waist/hip ratio, percentage of body fat and fibrosis progression rate were calculated. Applied dose of interferon and ribavirin, platelet and neutrophil count and hemoglobin level were measured.

RESULTS: A sustained virological response (SVR) was significantly associated with IL28B polymorphism (CC *vs* TT allele: odds ratio (OR), 25; CC *vs* CT allele: OR, 5.4), inflammation activity (G < 1 *vs* G > 1: OR, 3.9), fibrosis (F < 1 *vs* F > 1: OR, 5.9), platelet count (> 200 × 10^9/L *vs* < 200 × 10^9/L: OR, 4.7; OR in patients with genotype CT: 12.8), fatty liver (absence *vs* presence of steatosis: OR, 4.8), insulin resistance index (< 2.5 *vs* > 2.5: OR, 3.9), and baseline HCV viral load (< 10^6 IU/mL *vs* > 10^6 IU/mL: OR, 3.0). There was no association with age, sex, aminotransferases activity, body mass index, waist/hip ratio, or percentage body fat. There was borderline significance (P = 0.064) of increased fibrosis in patients with the TT allele, and no differences in the insulin resistance index between groups of patients with CC, CT and TT alleles (P = 0.12). Spearman's rank correlation coefficient between insulin resistance and stage of fibrosis and body mass index was r = 0.618 and r = 0.605, respectively (P < 0.001). Significant differences were found in the insulin resistance index (P = 0.01) between patients with and without steatosis. Patients with the CT allele and absence of a SVR had a higher incidence of requiring threshold dose reduction of interferon (P = 0.07).

CONCLUSION: IL28B variation is the strongest host factor not related to insulin resistance that determines outcome of antiviral therapy. Baseline platelet count predicts the outcome of antiviral therapy in CT allele patients.

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Key words: IL28B polymorphism; Hepatitis C; Fibrosis; Progression; Peg-interferon alpha; Sustained viral response

Peer reviewer: Takumi Kawaguchi, MD, PhD, Department of Digestive Disease Information and Research, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan

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INTRODUCTION

Investigations on genetic determinants of chronic hepatitis C (CHC) established that a single nucleotide polymorphism (SNP) in the interleukin (IL)-28B gene promoter region affected the spontaneous and induced clearance of hepatitis C virus (HCV)[1-5]. Among 500 000 genetic variants which were analyzed genome-wide, a few associated with the virologic response were identified, and showed variable frequency and importance across human ethnic groups[3,5-7]. The mechanism by which SNPs influence the outcome of HCV infection and its treatment is not clear. It is suggested that regulation of the promoter region of IL28B in antiviral activity may also affect two other genes belonging to interferon (IFN)-λ family encoded in this region[6,8-12]. According to some authors, IL28B SNPs are merely indicators of more complex mechanisms of CHC pathogenesis[13]. Some factors which determine the efficacy of antiviral therapy influence the course of CHC[14]. The impact of SNPs within the IL28B region on spontaneous and induced HCV clearance suggests the possibility that polymorphism affects the chronic phase of hepatitis. On the basis of previous research, there is no clear evidence that there is a correlation between IL28B SNPs and progression of untreated CHC. In a meta-analysis by Romero-Gomez *et al*[15], an effect of IL28B polymorphism on the progression of fibrosis was disputed. Research evaluating liver cirrhosis and fibrosis progression after liver transplantation in HCV-infected patients indicated a connection with IL28B SNPs[16,17].

However, about 50% of patients with a sustained virological response (SVR) do not carry the favorable IL28B alleles[15]. The factors which increase the chance of a therapeutic response in these patients are not yet known. A detailed analysis of the course of therapy of CHC with pegylated IFNα and ribavirin (RBV) in the presence of a hazardous IL28B allele might better delineate the clinical characteristics of this difficult-to-treat group of patients.

The aim of this study was to determine the impact of IL28B polymorphism on the progression of CHC, in order to identify predictors of effective therapy with IFN and RBV in the group of patients with unfavorable IL28B polymorphism.

Table 1 Characteristics of patients

IL28B polymorphism	*n* (%)	SVR (%)	Gender (M/F)	Age (yr)	BMI (kg/m^2)
CC	13 (20)	77			
CT	34 (53)	38			
TT	17 (27)	6			
All	64	39	27/37	44 ± 12	27 ± 5

The values of BMI and age given as arithmetic mean and standard deviation. CC, CT and TT: Single nucleotide polymorphisms at location *rs12979860* IL28B; SVR: Sustained virological response; BMI: Body mass index; M/F: Male/female.

MATERIALS AND METHODS

Sixty-four patients of European origin (Caucasians) with CHC due to HCV genotype 1 were included in the present study. Patients were treated with standard antiviral therapy with pegylated IFN 2a or 2b and RBV according to standard criteria of inclusion and exclusion. A liver biopsy was performed before treatment, and HCV viral load (COBAS® TaqMan® HCV 2.0) and insulin resistance index (HOMA-IR: homeostasis model of assessment) was determined.

The histopathological assessment of necroinflammatory grade and fibrosis stage were scored using the METAVIR system, and the presence of fatty liver disease was taken into consideration. The course of therapy was monitored on the basis of early virologic response (EVR), end of treatment response (ETR) and SVR.

The presence of the IL28B-related SNP *rs 12979860* variant was determined in whole blood samples. Genomic DNA was isolated from peripheral blood samples using proteinase K ion exchange column extraction kit (A&A Biotechnology, Gdynia, Poland). An amplification product of 190 bp length was obtained using standard polymerase chain reaction with the following primers: 5'- GCC TCT TCC TCC TGC GGG ACA AG and 5'- GCG CGG AGT GCA ATT CAA CCC T. Following digestion with *Bsh*1236I (*Bst*UI) restriction endonuclease, products were separated using agarose gel electrophoresis. The variant C was digested by the enzyme into fragments. All laboratory procedures were performed on blinded samples.

Patient age, sex, measurable anthropometric parameters such as waist, waist/hip ratio, body mass index (BMI) and percentage of body fat from MALTRON® measurements were analyzed in the present study.

Based on estimated duration of infection, the fibrosis progression rate was defined as the ratio between fibrosis stage and HCV duration. The serum aminotransferase levels prior to the start of treatment and after 72 wk was assessed. Patient characteristics are presented in Table 1.

The complete blood count at baseline and its changes during the course of therapy were determined. Thresholds for drug dose reduction were established: white blood cells < 750 cells/μL platelets < 70 000/μL, and

Table 2 Odds ratios of sustained virological response, depending on type of polymorphism of IL28B, inflammation activity, fibrosis, fatty liver, baseline hepatitis C virus viral load, the level of platelets and insulin resistance

Features affecting SVR	*P* value	OR	95% CI
CC *vs* TT	< 0.001	25	3.5-177.5
Platelets CT	0.002	12.8	1.8-42.6
Staging	0.008	5.9	1.5-23.8
CC *vs* CT	0.018	5.4	1.2 -23.3
Fatty liver	0.014	4.8	1.2-13.6
Platelets	0.005	4.7	1.3-17.3
Grading	0.026	3.9	1.1-13.4
Insulin resistance	0.029	3.9	1.1-13.8
HCV viremia	0.038	3	1-8.8

Difference in the risk of a SVR between patients with the CC allele and TT and CT alleles, low and high activity of the inflammatory process (G < 1 *vs* G > 1, according to METAVIR), little and advanced fibrosis (F < 1 *vs* F > 1, according to METAVIR), low and high hepatitis C viral load (< 10^6 IU/mL *vs* > 10^6 IU/mL), and absence or presence of fatty liver, high and low platelet count (> 200 × 10^9/L *vs* < 200 × 10^9/L), low or high insulin resistance index (< 2.5 *vs* > 2.5). Platelets CT: OR of SVR in patients with genotype CT with normal *vs* low platelets. SVR: Sustained virological response; CI: Confidence interval; HCV: Hepatitis C virus; OR: Odds ratio.

hemoglobin < 10 g/L. For each patient the dose of IFN and RBV used in the therapy was established and expressed as a percentage of the recommended dosage. The significance of differences in the clinical parameters as well as anthropometric, biochemical, histological and virological features between patients with and without a SVR was determined based on analysis of variance Kruskal-Wallis rank sum, Mann-Whitney *U* and χ^2 tests. A similar analysis was performed on patients only with the IL28B CT genotype. In patients with CC and TT genotypes, analysis of the diversity of the SVR was not performed because of the disproportionate distribution and small number of patients who did or did not achieve a SVR (Table 1).

Using the Kruskal-Wallis rank sum test, we assessed variations in the investigated clinical features between patients with different IL28B polymorphisms. In the logistic regression model we evaluated the odds ratio (OR) and 95% confidence interval (CI) of SVR, depending on the degree of inflammatory-necrotic activity, fibrosis scores, platelet count, baseline viral load, insulin resistance index (IR index) and IL28B polymorphisms (Table 2). The OR was determined against cases of high inflammatory-necrotic activity, above grade 1 in the METAVIR score, advanced fibrosis according to METAVIR F > 1, presence of steatosis, viral load > 1 × 10^6 IU/mL, alleles TT and CT, baseline platelet count < 200 × 10^9/L and IR index > 2.5.

The study was performed according to ethical guidelines of the 1975 Declaration of Helsinki. All patients were informed about the study, and provided written informed consent.

RESULTS

In the Mann-Whitney *U* test, differences were found in inflammatory-necrotic activity ($P = 0.011$), stage of fibrosis ($P = 0.009$), fibrosis progression rate ($P = 0.015$), EVR and ETR ($P = 0.00001$), baseline viral load ($P = 0.09$), reduction in viral load at 3 mo of therapy ($P = 0.0004$) and the presence of a biochemical response after completion of therapy ($P = 0.03$) between the patients who achieved a SVR and those who did not. In the group of patients who achieved a SVR, a lower incidence of liver steatosis was observed significantly (χ^2 test, $P = 0.062$). In the χ^2 test, there were no significant differences between patients with and without SVR as regards sex, age, comorbidities, and anthropometric factors. Differences in the parameters mentioned above were not observed among the three groups selected on the basis of the IL28B polymorphism. The χ^2 test showed differences in the rate of achievement of EVR ($P = 0.02$), ETR ($P = 0.069$) and SVR ($P = 0.001$) between groups of patients with CC, CT and TT alleles. The Kruskal-Wallis rank sum test showed differences in the size of the reduction of viral load after 3 mo of therapy ($P = 0.019$) between groups of patients with CC, CT and TT alleles. The OR revealed that patients with the CC allele had a 25-fold greater chance of achieving a SVR compared with those with the TT allele ($P < 0.001$) and a 5.4-fold higher chance compared with those with the CT allele ($P = 0.018$) (Table 2). Patients with low liver inflammation had a 3.9 times greater probability of achieving a SVR ($P = 0.026$), and patients with no or little fibrosis had 5.9 times greater probability ($P = 0.008$). HCV viral load < 1 × 10^6 IU/mL increased the chance of achieving a SVR by 3-fold ($P = 0.038$), and an IR index < 2.5 increased the chance by 3.9-fold ($P = 0.029$). The Kruskal-Wallis rank sum test showed no differences in the IR index between groups of patients with CC, CT and TT alleles ($P = 0.12$). Spearman's rank correlation coefficient between IR index and stage of fibrosis and BMI was $r = 0.618$ and $r = 0.605$, respectively ($P < 0.001$). In the Mann-Whitney *U* test, differences were found in the IR index ($P = 0.005$) between patients with and without steatosis. The Kruskal-Wallis rank sum test revealed borderline significance ($P = 0.064$) of increased fibrosis in patients with the TT allele (Figure 1).

The Mann-Whitney *U* test established that patients who did not achieve a SVR had low platelet levels at baseline ($P = 0.001$) compared with subjects with a SVR. A similar trend was also found for neutrophils ($P > 0.05$). Patients without SVR bearing the CT allele, were characterized by lower baseline platelet count ($P = 0.003$) and decreased ($P = 0.02$) drop of platelet count upon treatment. Decreased platelet levels in patients with the CT allele and absence of a SVR were accompanied by a higher incidence of requiring a dose reduction of IFN ($P = 0.07$). No differences in IFN and RBV dosage in patients with the CT allele or other allelic variants were found between groups with or without a SVR.

DISCUSSION

The present study observed an impact of IL28B SNPs

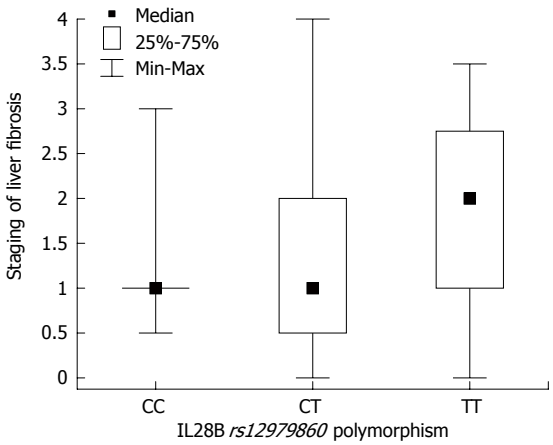


Figure 1 Liver fibrosis stage (by METAVIR) is increased in patients with the TT allele of the *rs12979860* region in the IL28B gene compared with patients with CC and CT alleles using the Kruskal-Wallis test (*P* = 0.06).

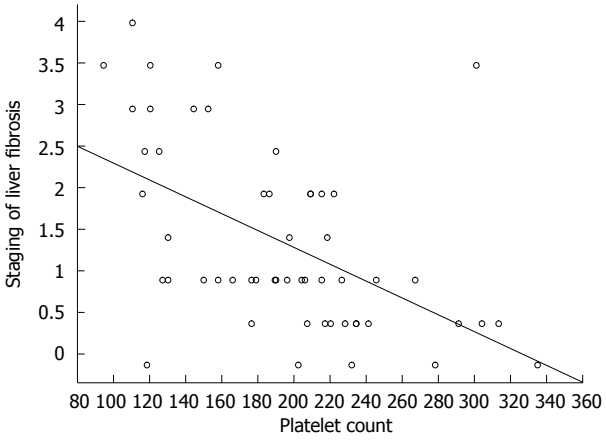


Figure 2 Correlation between liver fibrosis stage (by METAVIR) and the platelet count (10^9/L). Spearman's correlation coefficient r = -0.565, P < 0.001.

on the efficacy of IFN and RBV in CHC, which was consistent with the results from previous research. In patients with TT and CT genotypes, greater severity of liver fibrosis and necroinflammation, presence of liver steatosis, higher HCV viral load, and lower baseline platelets, had a reduced chance of therapeutic success. In the OR of the SVR, the greatest differences concerned the IL28B polymorphism between CC and TT alleles. The difference was more than 4-fold for fibrosis in the comparison of the CC allele with the CT allele. For liver steatosis, low platelet level, necroinflammatory grade, IR index and initial viral load of HCV, the difference was 5-8-fold (Table 2). The results confirm that polymorphism in the IL28B is significantly related to the outcome of antiviral therapy of CHC.

The mechanism by which genetic factors influence the progression of fibrosis in CHC remains unclear[18-21]. In the present study, patients with the TT genotype had a more active state of necroinflammation in the histological analysis, and borderline significance (P = 0.06) of greater severity of fibrosis (Figure 1). This observation is consistent with previous results describing a higher incidence of TT or CT allele in cases of cirrhosis and faster fibrosis progression in HCV-infected liver transplant recipients and liver from donors with the TT genotype[16,17]. Taking into consideration that the correlation between the degree of fibrosis and IL28B was not statistically significant and there was no association with fibrosis progression rate, it was not possible to confirm the impact of the genotype on the progress of CHC. Thus, IL28B polymorphism is probably not the factor which directly affects the outcome of untreated CHC.

The correlation between insulin resistance and stage of fibrosis, presence of the liver steatosis, efficacy of IFN/RBV therapy and higher BMI observed in the present study is consistent with the results from previous observations[22-27]. No statistically significant correlation between insulin resistance and IL28B polymorphism was observed, indicating that there are different mechanisms by which both factors can influence the course and therapy of CHC.

In the present study 60% of patients with SVR had TT (8%) or CT (52%) genotypes, which are not known factors associated with higher IFN responsiveness. These values are similar to those described in previous reports[15]. Analysis of the differences in the course of therapy in patients with the CT allele and a SVR revealed higher baseline platelet and neutrophil levels. These factors were markedly reduced in the group of patients with therapeutic failure. The present results are consistent with existing data, where the platelet level was a predictor of a rapid virological response[28]. In this study, a low baseline platelet count was significantly associated with the need for IFN dose reduction in the group without a SVR. In the group of patients with the CT allele, the platelet count was the only factor differentiating patients with and without a SVR. The chance of a SVR was 8-fold higher in patients with the CT allele and higher platelet count compared with patients with low levels of platelets.

The platelet level is one of the factors used to estimate the risk of the presence of advanced liver fibrosis[29]. In the present study, the platelet level also correlated with the stage of liver fibrosis (Figure 2). More severe fibrosis, and a lower baseline platelet level increased the risk of requiring a reduction in IFN dose and subsequent reduction in the chance of achieving a SVR[13]. Assessment of the platelet level and the IL28B polymorphism can complement the decision-making algorithm for a patient's eligibility for antiviral therapy.

Differentiation of SNPs in the IL28B promoter region is the strongest factor determining the effectiveness of therapy with IFN and RBV. Despite the greater severity of fibrosis in patients with the TT genotype, there was no additional evidence of an IL28B SNP associated with progression of CHC. In patients with the IL28B CT allele, a low baseline platelet count is associated with a requirement to reduce IFN and a decreased chance of obtaining a SVR.

COMMENTS

Background

Ineffective therapy for chronic viral hepatitis type C (CHC) determines the progression of liver disease to cirrhosis and primary hepatocellular carcinoma. Single nucleotide polymorphism (SNP) in the IL28B promoter gene region determines spontaneous and induced clearance of hepatitis C virus.

Research frontiers

Clinical aspects of the impact of IL28B SNPs on the efficacy of antiviral therapy and progression of CHC are not fully recognized. In the present study, potential factors influencing the efficacy of therapy in relation to IL28B polymorphism were investigated.

Innovations and breakthroughs

The results of the study confirm a significant role of SNPs in the IL28B gene among different predictive factors of a sustained virological response (SVR). The impact of IL28B SNPs on the SVR is not related to insulin resistance. In the case of the CT allele, a low baseline platelet count is associated with a reduced chance of a SVR. The phenomenon is connected with the stage of liver fibrosis and need for a reduction of interferon dose during therapy. The results suggest that the progression of CHC is faster in patients with the TT genotype.

Applications

Results of the study can complement the decision-making algorithm for eligibility of patients with CHC for antiviral therapy.

Terminology

Single nucleotide polymorphism is a variation of a single nucleotide usually found in non-coding microsatellite fragments of the genome. It may serve as the fingerprint for predicting susceptibility toward disease. IL28B SNPs include different variants localized in the gene promoter region, among which the most common in clinical practice is C/T SNP of *rs 12979860*.

Peer review

This study examined the association of SNPs of the IL28B, rs12979860 on progression of CHC. They found that SNPs of IL28B had any impact on CHC progression, in spite of apparently more advanced fibrosis among patients carrying TT genotype. In addition, they revealed that, in patients with the CT allele IL28B, low baseline platelets count is linked to the reduction of IFN and decreased chance of reaching the SVR. This study demonstrated a new characteristic of IL-28b in CHC.

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BRIEF ARTICLE

Analysis of colonoscopic perforations at a local clinic and a tertiary hospital

Toshihiko Sagawa, Satoru Kakizaki, Haruhisa Iizuka, Yasuhiro Onozato, Naondo Sohara, Shinichi Okamura, Masatomo Mori

Toshihiko Sagawa, Satoru Kakizaki, Shinichi Okamura, Masatomo Mori, Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan
Toshihiko Sagawa, Center of Gastroenterology, Maebashi Red Cross Hospital, Maebashi, Gunma 371-0014, Japan
Haruhisa Iizuka, Yasuhiro Onozato, Naondo Sohara, Department of Endoscopy and Endoscopic Surgery, Shirakawa Clinic, Maebashi, Gunma 371-0051, Japan
Author contributions: Sagawa T and Iizuka H designed the study and collected the data; Kakizaki S analyzed the data and wrote the manuscript; Onozato Y and Sohara N collected the data; Okamura S and Mori M offered vital suggestions.
Correspondence to: Satoru Kakizaki, MD, PhD, Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan. kakizaki@showa.gunma-u.ac.jp
Telephone: +81-27-2208127 Fax: +81-27-2208136
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Abstract

AIM: To define the clinical characteristics, and to assess the management of colonoscopic complications at a local clinic.

METHODS: A retrospective review of the medical records was performed for the patients with iatrogenic colon perforations after endoscopy at a local clinic between April 2006 and December 2010. Data obtained from a tertiary hospital in the same region were also analyzed. The underlying conditions, clinical presentations, perforation locations, treatment types (operative or conservative) and outcome data for patients at the local clinic and the tertiary hospital were compared.

RESULTS: A total of 10 826 colonoscopies, and 2625 therapeutic procedures were performed at a local clinic and 32 148 colonoscopies, and 7787 therapeutic procedures were performed at the tertiary hospital. The clinic had no perforations during diagnostic colonoscopy and 8 (0.3%) perforations were determined to be related to therapeutic procedures. The perforation rates in each therapeutic procedure were 0.06% (1/1609) in polypectomy, 0.2% (2/885) in endoscopic mucosal resection (EMR), and 3.8% (5/131) in endoscopic submucosal dissection (ESD). Perforation rates for ESD were significantly higher than those for polypectomy or EMR ($P < 0.01$). All of these patients were treated conservatively. On the other hand, three (0.01%) perforation cases were observed among the 24 361 diagnostic procedures performed, and these cases were treated with surgery in a tertiary hospital. Six perforations occurred with therapeutic endoscopy (perforation rate, 0.08%; 1 per 1298 procedures). Perforation rates for specific procedure types were 0.02% (1 per 5500) for polypectomy, 0.17% (1 per 561) for EMR, 2.3% (1 per 43) for ESD in the tertiary hospital. There were no differences in the perforation rates for each therapeutic procedure between the clinic and the tertiary hospital. The incidence of iatrogenic perforation requiring surgical treatment was quite low in both the clinic and the tertiary hospital. No procedure-related mortalities occurred. Performing closure with endoscopic clipping reduced the C-reactive protein (CRP) titers. The mean maximum CRP titer was 2.9 ± 1.6 mg/dL with clipping and 9.7 ± 6.2 mg/dL without clipping, respectively ($P < 0.05$). An operation is indicated in the presence of a large perforation, and in the setting of generalized peritonitis or ongoing sepsis. Although we did not experience such case in the clinic, patients with large perforations should be immediately transferred to a tertiary hospital. Good relationships between local clinics and nearby tertiary hospitals should therefore be maintained.

CONCLUSION: It was therefore found to be possible to perform endoscopic treatment at a local clinic when sufficient back up was available at a nearby tertiary hospital.

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Key words: Colonoscopy; Colon perforation; Endoscopic clipping; Endoscopic submucosal dissection; Endoscopic mucosal resection; Polypectomy

Peer reviewers: Dr. Daniel R Gaya, Department of Gastroenterology, Glasgow Royal Infirmary, Castle Street, Glasgow G4 0SF, United Kingdom; Maha Maher Shehata, Professor, Internal Medicine, Mansoura University, Medical Specialized Hospital, Mansoura 35516, Egypt

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INTRODUCTION

Colonoscopy is widely used for the diagnosis, treatment, and follow-up of colorectal diseases[1,2]. The improvement of the equipment and increased needs for screening colonoscopy has increased the number of colonoscopies rapidly not only at hospitals but also in clinics. It is convenient for patients to have lesions treated when they are identified by either a routine-check up or diagnostic endoscopy. Therefore, therapeutic endoscopy is sometimes needed at a local clinic, including on the day surgery to perform polypectomy. The occurrence of complications in such cases negatively affects the quality life of these patients. Bleeding after polypectomy is the most common complication[3]. However, the development of endoscopic clipping prevents the occurrence of bleeding after polypectomy[4]. Therefore, cases that need surgical treatment for bleeding after therapeutic procedures are quite rare. Although perforation occurs less often, it is more problematic than bleeding and should be given the most attention[5,6]. Perforations sometimes require surgical intervention and will decrease the patients' quality of life. Several large, retrospective studies have determined perforation incidences of 0.02%-0.8% and 0.15%-3% for diagnostic and therapeutic colonoscopy, respectively[6-11].

Surgery has been the mainstream treatment for iatrogenic perforation[12-14]. Surgical treatment for iatrogenic perforation should be avoided at local clinics because most such clinics do not have the appropriate equipment for such surgery. As a result, the performance of therapeutic endoscopy has so far not become common at local clinics. However, the use of endoscopic clipping to prevent the leakage of intestinal contents can circumvent the need for surgery[15,16]. Although surgical treatment should be selected when it is needed, the use of endoscopic clipping could potentially extend the therapeutic indications for such treatments at local clinics.

The aim of this study was to determine the incidences, clinical presentations, and management of iatrogenic perforations that occurred after diagnostic and therapeutic colonoscopy at a local clinic. This study compared the data between a local clinic and a tertiary hospital. Finally, the optimal strategies for performing therapeutic endoscopy and steps for dealing with complications at local clinics are also discussed.

MATERIALS AND METHODS

This study retrospectively reviewed the patient database of colonoscopies, and therapeutic procedures at Shirakawa Clinic (Maebashi, Japan) between April 2006 and December 2010. The data from Maebashi Red Cross Hospital (Maebashi, Japan), a tertiary hospital in the same region were also analyzed and compared between January 1996 and December 2010. Endoscopies at Shirakawa Clinic and Maebashi Red Cross Hospital are performed or supervised by staff gastroenterologists or fellows. The Shirakawa Clinic has 19 inpatient beds and appropriate management, such as drip infusion, can be easily carried out. However, the performance of either surgery or intensive care is restricted and patients required such case are therefore referred to the Maebashi Red Cross Hospital which is a tertiary hospital. Patients that required treatment for an iatrogenic colon perforation during the study period were analyzed. The underlying conditions, clinical presentations, perforation locations, treatment types (operative or conservative), and outcome data were analyzed. Possible complications were explained to all patients before the procedures, and all provided their written consent. Outpatients were informed to contact the clinic if they experienced any post-procedural abdominal distension or pain. This study was approved by the institutional ethical committee (No. SC2011/003; date, December 10, 2010).

Endoscopic procedures

The device for diagnostic endoscopy was a single-channel endoscope (CF260AI, Olympus Optical Co, Tokyo, Japan). A single-channel endoscope (GIF230 and/or CF260AI, Olympus) with a hood and a high-frequency generator with an automatically controlled system (Erbotom ICC200 or VIO 300D, ERBE, Tuebingen, Germany) were used for the endoscopic submucosal dissection (ESD) procedure. The patients principally received 24 mg sennoside the night before the examination for bowel preparation, and drank 200 mL polyethylene glycol (PEG) solution (Niflec®, Ajinomoto Pharma Co., Ltd., Tokyo, Japan) every 10 min on the examination day, for a total intake of 2000 mL PEG solution. Polypectomy and endoscopic mucosal resection (EMR) were performed as usual[17,18]. The ESD procedure was performed as described previously[19-21]. Abdominal X-rays were routinely performed after therapeutic procedures to check the perforation. Patients without complications were permitted to take soft food the day after the therapeutic procedures. Hemoclips (HX-600-135 and HX-600-090L, Olympus) were used for the endoscopic closure of any perforation.

Statistical analysis

The data were expressed as the mean ± SD. The data were analyzed using the Fisher's exact probability test,

Table 1 Frequency of perforation depending on the type of endoscopic procedure

		Total	Diagnostic	Therapeutic	Polypectomy	EMR	ESD
Shirakawa Clinic between April 2006 and December 2010							
Number	*n*	10 826	8201	2625	1609	885	131
Perforation	*n* (%)	8 (0.07)	0 (0)	8 (0.3)	1 (0.06)	2 (0.2)	5 (3.8)
Maebashi Red Cross Hospital between January 1996 and December 2010							
Number	*n*	32 148	24 361	7787	5500	2244	43
Perforation	*n* (%)	9 (0.03)	3 (0.01)	6 (0.08)	1 (0.02)	4 (0.17)	1 (2.3)

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

Table 2 The cases with iatrogenic perforation at the Shirakawa Clinic

Case	Age	Gender	Procedure	Site of perforation	Disease	Discovery of perforation	Abdominal pain	Peritonitis sign	Extrabowel gasses	Treatment	Max body temperature (℃)	CRP (mg/dL)	Cessation of food intake (d)	Intravenous antibiotics treatment (d)	Total hospital stay (d)
1	71	F	EMR	Ascending	Adenocarcinoma	Just after procedure	+	No	Free air	Conservative (with clipping)	36.9	4.4	1	6	7
2	57	M	ESD	Ascending	Adenocarcinoma	During	-	No	Free air	Conservative (with clipping)	36.8	0.9	1	4	9
3	69	F	ESD	Caecum	Adenoma	During	-	No	PP[1]	Conservative (with clipping)	37.5	3.8	2	8	13
4	66	M	ESD	Rectm (Rb)	Adenoma	Just after procedure	-	No	PP	Conservative (without clipping)	37.9	17.9	1	6	12
5	80	M	ESD	Caecum	Adenoma	Just after procedure	-	No	Free air	Conservative (without clipping)	37.0	4.2	2	6	10
6	68	M	Polypectomy	Transverse	Adenoma	1 d after procedure	+	Located	Free air	Conservative (without clipping)	36.9	11	4	7	10
7	64	M	EMR	Ascending	Adenocarcinoma	2 d after procedure	+	Located	Free air	Conservative (without clipping)	36.9	5.5	4	6	11
8	64	F	ESD	Ascending	Adenocarcinoma	During	+	Located	Free air	Conservative (with clipping)	37.2	2.4	3	5	11

[1]Pneumoretroperitoneum. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; CRP: C-reactive protein; M: Male; F: Female.

and Mann-Whitney's *U*-test. A *P* value less than 0.05 was considered to be significant.

RESULTS

Patients and characteristics

A total of 10 826 colonoscopies, and 2625 therapeutic procedures were performed at the Shirakawa Clinic between April 2006 and December 2010. Eight (0.07%) perforations were attributed to endoscopy (Table 1). There were no perforation cases for diagnostic procedures. On the other hand, a therapeutic procedure was performed in the 8 perforation cases (perforation rate, 0.3%; 1 per 328 procedures). The perforation rates for specific procedure types were 0.06% (1 per 1609) for polypectomy, 0.2% (1 per 443) for EMR, and 3.8% (1 per 26) for ESD. Perforation rates for ESD were significantly higher than those for polypectomy or EMR ($P < 0.01$). The cases with iatrogenic perforation are shown in Table 2. The study group included 3 females and 5 males aged from 57 years to 80 years of age (mean age 67.4 ± 6.6 years).

A total of 32 148 colonoscopies, and 7787 therapeutic procedures were performed in Maebashi Red Cross Hospital, and 9 (0.03%) perforations were attributed to endoscopy (Table 1). There were 3 (0.01%) perforation cases among the diagnostic procedures. Six perforations occurred with therapeutic endoscopy (perforation rate, 0.08%; 1 per 1298 procedures). Perforation rates for specific procedure types were 0.02% (1 per 5500) for polypectomy, 0.17% (1 per 561) for EMR, 2.3% (1 per 43) for ESD. The patients included 3 females and 6 males from 59 years to 81 years of age (mean age 69.2 ± 8.5 years). The cases with perforation during diagnostic endoscopy were complicated with diverticulitis, radiation colitis and amyloidosis, respectively. These 3 perforations during diagnostic endoscopy were treated by surgery. There were no significant differences in the perforation rates for each therapeutic procedure between the local clinic and the tertiary hospital.

Management and outcomes of perforations in the clinic

Three of 8 cases (37.5%) perforations were detected during the endoscopic procedure due to the visualization of a tear in the serosa. Six (75%) patients were diagnosed to have a perforation within 24 h of colonoscopy. Two patients were diagnosed to have a perforation more than 24 h later. These cases were considered to be delayed perforation caused by electrocautery. Four (50%) of 8 patients had been undergone clipping. Six (75%) patients had free intraperitoneal air and 2 patients showed pneumoretroperitoneum by abdominal radiography or computed tomography (CT). There were no symptoms of peritonitis in 5 patients and 3 patients showed localized peritonitis. All patients were managed conservatively because there no progression to peritonitis symptoms, Conservative treatment included the withholding of oral intake, hydration, intravenous antibiotics and serial abdominal examinations.

The mean maximum C-reactive protein (CRP) titer was 6.3 ± 5.6 mg/dL (range 0.9-17.9 mg/dL) and maximum body temperature was 37.1 ± 0.4 ℃ (range: 36.8-37.9 ℃). The mean fasting period for these patients was 2.3 ± 1.3 d (range: 1-4 d) and the mean hospital stay following perforation was 10.4 ± 1.8 d (range: 7-13 d). The points of references used to discontinue fasting were relief of abdominal pain, improvement of leukocytosis. The mean duration of total intravenous antibiotic treatment was 6.0 ± 1.2 d (range 4-8 d). Flomoxef sodium (FMOX) was administered in all cases. When fasting had been completed, oral ciprofloxacin was prescribed.

The mean maximum CRP titer was 2.9 ± 1.6 mg/dL with clipping and 9.7 ± 6.2 mg/dL without clipping, respectively. The mean maximum CRP titer was significantly lower in the patients with clipping ($P < 0.05$). The duration of fasting with and without clipping were 1.8 ± 1.0 d and 2.8 ± 1.5 d, respectively. The duration of intravenous antibiotics treatment with and without clipping was 5.8 ± 1.7 d and 6.3 ± 0.5 d, respectively. The duration of total the hospital stay with and without clipping were 10.0 ± 2.6 d and 10.8 ± 1.0 d, respectively. There were no significant differences in the total hospital stay, duration of fasting, dose of intravenous antibiotics between those that had or had not undergone clipping because of the small number of patients with perforation. However, the duration of fasting, dose of intravenous antibiotics and total hospital stay tended to be shorter when clipping was successful in comparison to when clipping was not performed.

Representative cases

Perforation discovered during the therapeutic procedure and treated with clipping: The case was 57-year-old male with no major complications. Perforation was observed during an ESD procedure for ascending colon adenocarcinoma (Figure 1A). Carbon dioxide inflation was used during ESD. Endoscopic clipping closed the small tear of the serosa. Abdominal X-ray showed a small amount of extra-bowel gasses. This case was treated conservatively. FMOX was administered for 4 d. The patient fasted for 1 d and food intake was started at day 3. The patient was discharged at day 9.

Perforation discovered more than 24 h after therapeutic procedure and treated without clipping: The case was a 64-year-old male with a past history of myocardial infarction. Abdominal pain was appeared after 2 d an EMR procedure for ascending colon adenoma. Abdominal XP and CT findings revealed free air (Figure 1B). This case was treated conservatively because the symptoms of peritonitis were localized. FMOX was administered for 6 d. The patient fasted for 4 d and food intake was started at day 6. The patient was discharged on day 11.

Perforation discovered just after a therapeutic procedure and treated with suture clippings: The case was a 71-year-old female complicated with hypertension. This case was treated by usual air inflation during the EMR procedure. Abdominal XP and CT findings revealed free air just after the EMR procedure for ascending colon adenoma (Figure 1C). Inflation was changed to CO_2 inflation and endoluminal repair with suture clipping was performed. FMOX was administered for 6 d. The patient fasted for 1 d and food intake was started on day 3. The patient was discharged on day 7.

Trouble shooting for perforation at a local clinic

A management algorithm for colonoscopic bowel perforation at a clinic is shown in Figure 2. A minor colon injury that is recognized during colonoscopy should be treated by changing to CO_2 inflation, endoluminal repair with clips and further conservative treatment could avoid immediate surgical intervention. Delayed endoscopic repair with clipping should be considered only if the condition of the patient is stable and a specific site is highly suspected. This latter recommendation should be useful when the perforation is suspected in the rectosigmoid area, because perforations in this region are easily located on scope reinsertion. An operation is indicated in the presence of a large perforation, and in the setting of generalized peritonitis or ongoing sepsis. Such patients should be immediately transferred to a tertiary hospital. A good relationship between local clinics and nearby tertiary hospitals should therefore be maintained.

DISCUSSION

The perforation risks of diagnostic and therapeutic colonoscopy are 0.02%-0.8% and 0.15%-3%, respectively[6-11]. Large studies of iatrogenic perforation related to colonoscopy are shown in Table 3[6-11,22-24]. New therapeutic approaches including ESD techniques have become more popular and the risk of perforation is increased in comparison to conventional techniques such as polypectomy or EMR. The perforation risk of a therapeutic procedure is usually higher than that of a diagnostic procedure. Fortunately, there were no perforations during diagnostic procedures at the local clinic in the present study.

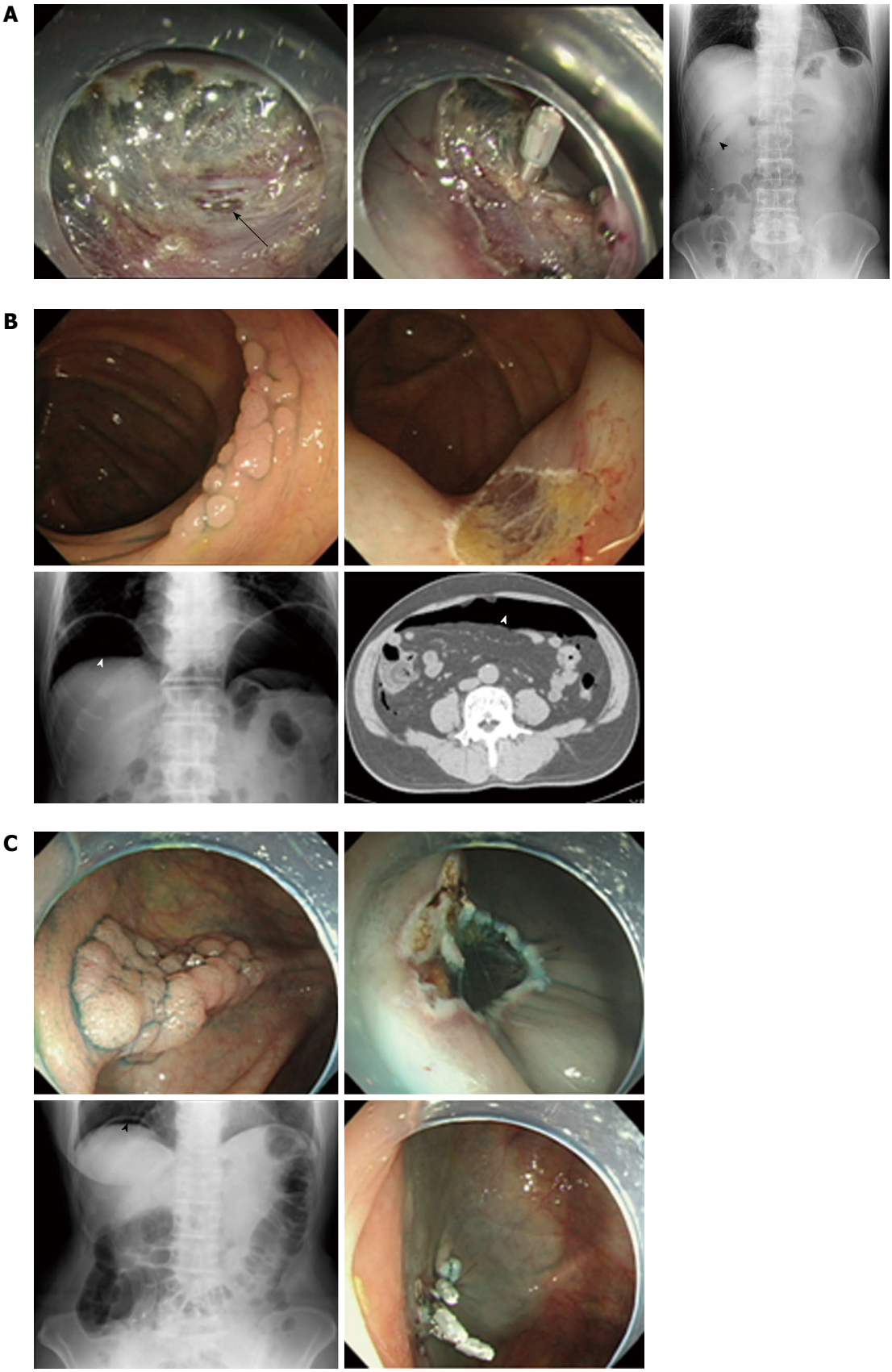


Figure 1 Representative cases of colonoscopic perforations. A: Case 1: Perforation discovered during the therapeutic procedure and treated with clipping; B: Case 2: Perforation discovered more than 24 h later after the therapeutic procedure and treated without clipping; C: Case 3: Perforation discovered just after the therapeutic procedure and treated with suture clipping.

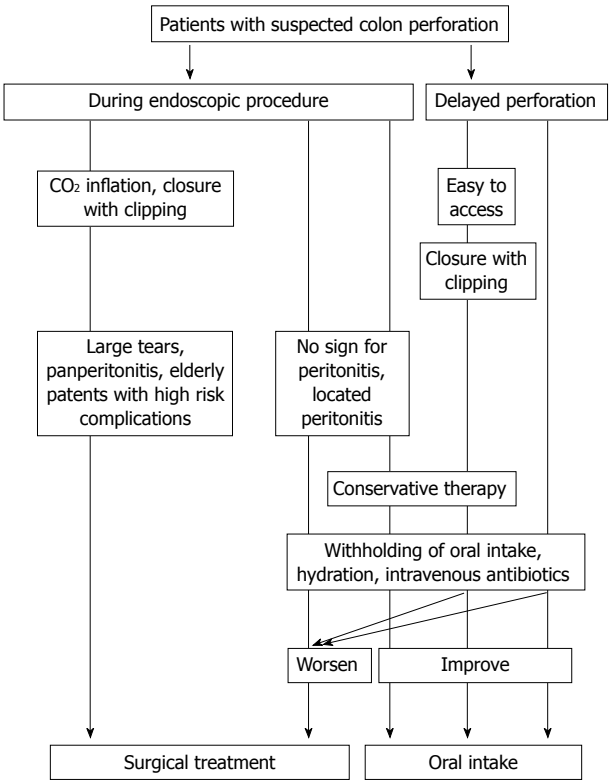


Figure 2 Trouble shooting for perforation at the local clinic.

Recent studies have demonstrated the possibility of endoscopic perforation closure using endoclips[25,26]. Closure by endoscopic clipping significantly reduced the maximum CRP titer in this study. Successful closure with endoscopic clipping in conservatively managed patients can reduce the fasting period, duration of intravenous antibiotic administration, and hospital stay[25,26]. As a result, successful clipping can improve patient quality of life and reduce medical costs. This study found no significant differences in the duration of fasting, intravenous antibiotics treatment, total hospital stay between those that had or had not undergone clipping. It may be due to the small number of patients with perforation.

Perforations associated with diagnostic procedures are usually due to applying pressure to the colonic wall, and they are noticed immediately. However, perforations that occur after therapeutic procedures are often diagnosed late. Ischemia of the colonic wall caused by electrical or thermal injury after electrocoagulation can cause a delayed perforation following therapeutic procedures[10]. Clipping can induce mucosal and submucosal healing and prevent fecal soiling of the peritoneal cavity when the perforation is small and significant colonic pathology does not exist. However, the application of clipping for perforation in delayed perforation still remains controversial. Delayed endoscopic repair with clipping should be considered only if the condition of the patient is stable and a specific site is highly suspected[6]. This recommendation is likely to be useful when the perforation is suspected to be in the rectosigmoid area, because perforations in this region are easily located on scope reinsertion[6].

Table 3 Large study of iatrogenic perforation related to colonoscopy *n* (%)

Ref.	Total	Diagnostic	Therapeutic	Polypectomy	EMR	ESD
Anderson *et al*[11]	59 987					
	22 (0.04)					
Cobb *et al*[10]	43 609					
	14 (0.03)					
Kaneko *et al*[24]	3 152 053	2 587 689	564 364	422 119	142 245	ND
	1387 (0.04)	568 (0.02)	819 (1.4)	621 (0.15)	198 (0.14)	ND
Iqbal *et al*[9]		78 702				
		66 (0.84)				
Tulchinsky *et al*[23]	120 067					
	7 (0.06)					
Lüning *et al*[7]		30 366				
		35 (0.12)				
Lüning *et al*[7]		433 816				
		393 (0.09)				
Taku *et al*[8]			15 160	8240	1906	43
			23 (0.15)	4 (0.05)	12 (0.63)	6 (14.0)
Kang *et al*[6]	44 534	37 762	6772			
	53 (0.12)	26 (0.07)	27 (0.40)			
Oka *et al*[22]			71 204	34 433	36 083	688
			62 (0.09)	6 (0.02)	33 (0.09)	23 (3.3)

Upper column: Number of cases; lower column: Perforation (%). EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; ND: No data.

The limitations of this study include the retrospective nature of the work and the inclusion of descriptions of local experience without the addition of new protocols. However, we believe that investigating a large number of patients at a local clinic will elucidate the role the local clinic plays in therapeutic endoscopies. Furthermore, selection bias between the two institutes of patients or endoscopists may have existed. The incidence of complications depends on the type of lesion or the skill of the endoscopist. Selection bias of patients or endoscopists is a problem to be evaluated in a future study.

Selective patients are likely to improve under conservative management involving hospitalization, intestinal rest, intravenous fluids, and antibiotics to limit peritonitis and allow the perforation to seal. However, conservative management requires careful observation with frequent and repeated abdominal exams. Patients successfully treated non-surgically must be clinically stable, and their abdominal symptoms should improve rapidly with no deterioration due to peritoneal signs[14]. A local clinic does not have any surgical options, so a good relationship and close contact with a tertiary hospital is needed. Patients must be immediately transferred to a tertiary hospital when either abdominal symptoms are observed or peritonitis worsens.

In conclusion, iatrogenic colonic perforation is a serious but uncommon complication of colonoscopy. However, surgery is not mandatory for perforations caused by therapeutic procedures, and endoscopic perforation closure using endoclips should be considered as a helpful adjunct to conservative treatment. It is possible to perform endoscopic treatment at a local clinic when there is appropriate back-up by a nearby tertiary hospital. Of course, close contact between local clinics and a tertiary hospital is essential.

COMMENTS

Background

The number of colonoscopies has increased rapidly, not only in hospitals, but also in clinics. The occurrence of complications negatively affects the quality life of patients. The aim of this study was to determine the incidence, clinical presentation and management of iatrogenic perforations that occurred after diagnostic and therapeutic colonoscopies at a local clinic.

Research frontiers

Data and management strategies regarding iatrogenic perforations at hospitals were reported. However, such reports from clinics were rare. Optimal strategies for performing therapeutic endoscopy and steps for managing complications at local clinics were also discussed.

Innovations and breakthroughs

This study investigated the incidence, management and outcomes of colonoscopic perforation in a local clinic. Performing endoscopic perforation closure using endoclips should be considered as a helpful adjunct to conservative treatment. It is possible to perform endoscopic treatment at a local clinic when appropriate back-up support at a nearby tertiary hospital is available.

Applications

The number of endoscopic treatments performed at local clinics will increase when appropriate back-up support at nearby tertiary hospitals is available.

Terminology

A colonoscopic perforation is a complication of diagnostic or therapeutic colonoscopy. Although perforation occurs infrequently, it is problematic and should be given proper attention. Perforations sometimes require surgical intervention, and will decrease a patient's quality of life.

Peer review

The study is well designed and the paper is well written. Although there is no novelty in the idea and only a description of the local experience is provided, the study is a nice piece of work that includes a large number of patients.

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BRIEF ARTICLE

High-risk esophageal varices in patients treated with locoregional therapy for hepatocellular carcinoma: Assessment with liver computed tomography

Hyojin Kim, Dongil Choi, Joon Hyeok Lee, Soon Jin Lee, Hangi Jo, Geum-Youn Gwak, Kwang Cheol Koh, Moon Seok Choi, Seonwoo Kim

Hyojin Kim, Dongil Choi, Soon Jin Lee, Hangi Jo, Department of Radiology and Center for Imaging Science, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea
Joon Hyeok Lee, Geum-Youn Gwak, Kwang Cheol Koh, Moon Seok Choi, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea
Seonwoo Kim, Biostatistics Unit, Samsung Biomedical Research Institute, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea
Author contributions: Choi D and Lee JH designed research; Choi D, Lee JH, Lee SJ, Jo H, Gwak GY, Koh KC, Choi MS and Kim S analyzed data; Kim H wrote the paper.
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Correspondence to: Dongil Choi, MD, Department of Radiology and Center for Imaging Science, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-Dong, Kangnam-Ku, Seoul 135-710, South Korea. dichoi@skku.edu
Telephone: +82-2-34102518 Fax: +82-2-34100084
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Abstract

AIM: To assess the diagnostic performance of follow-up liver computed tomography (CT) for the detection of high-risk esophageal varices in patients treated with locoregional therapy for hepatocellular carcinoma (HCC).

METHODS: We prospectively enrolled 100 patients with cirrhosis who underwent transcatheter arterial chemoembolization, radiofrequency ablation or both procedures for HCCs. All patients underwent upper endoscopy and subsequently liver CT. Three radiologists independently evaluated the presence of high-risk esophageal varices with transverse images alone and with three orthogonal multiplanar reformation (MPR) images, respectively. With endoscopic grading as the reference standard, diagnostic performance was assessed by using receiver operating characteristic (ROC) curve analysis.

RESULTS: The diagnostic performances (areas under the ROC curve) of three observers with transverse images alone were 0.947 ± 0.031, 0.969 ± 0.024, and 0.916 ± 0.038, respectively. The mean sensitivity, specificity, positive predicative value (PPV), and negative predicative value (NPV) with transverse images alone were 90.1%, 86.39%, 70.9%, and 95.9%, respectively. The diagnostic performances, mean sensitivity, specificity, PPV, and NPV with three orthogonal MPR images (0.965 ± 0.025, 0.959 ± 0.027, 0.938 ± 0.033, 91.4%, 89.5%, 76.3%, and 96.6%, respectively) were not superior to corresponding values with transverse images alone ($P > 0.05$), except for the mean specificity ($P = 0.039$).

CONCLUSION: Our results showed excellent diagnostic performance, sensitivity and NPV to detect high-risk esophageal varices on follow-up liver CT after locoregional therapy for HCC.

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Key words: Liver computed tomography; High-risk esophageal varices; Locoregional therapy; Hepatocellular carcinoma; Multiplanar reformation images

Peer reviewers: Eric R Kallwitz, Assistant Professor, University of Illinois, 840 S Wood Street, Chicago, IL 60612, United States; Hyo-Cheol Kim, Professor, Seoul National University Hospital, 28 Yongon-dong, Chongno-gu, Seoul 110-744, South Korea

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INTRODUCTION

More than one-half of patients with cirrhosis eventually develop esophageal varices[1]. Screening upper endoscopy is repeated in 1-3 years in order to evaluate esophageal varices in cirrhotic patients owing to the substantial morbidity and mortality related with variceal bleeding[2]. The presence of high-risk varices seen in up to 30% of patients with esophageal varices on endoscopy is considered as an indication for prophylaxis against variceal bleeding with either endoscopic variceal ligation (EVL) or the administration of β-blockers[1,3].

Of several recent studies that have suggested the usefulness of computed tomography (CT) scans to evaluate esophageal varices[4-8], one study[7] asserted that CT was a better initial screening modality when compared to endoscopy in terms of cost-effectiveness and patient preference. However, for only these reasons, the adoption of CT to replace ultrasound as the surveillance imaging technique in all cirrhotic patients is still subject to debate with the consideration of radiation hazards[9]. As CT is commonly used as a follow-up imaging modality in patients treated with locoregional therapy for hepatocellular carcinoma (HCC)[10], we supposed that replacement of CT for evaluating varices was more persuasive in such patients. The purpose of this study was to evaluate prospectively the diagnostic performance of radiologists for the detection of high-risk esophageal varices on regular follow-up liver multi-detector row computed tomography (MDCT) examinations in patients treated with locoregional therapy for HCC. We also assessed the usefulness of the addition of coronal and sagittal multiplanar reformation (MPR) images.

MATERIALS AND METHODS

Study design and patients

The Institutional Review Board approved the current study and all subjects provided informed consent. In this prospective study, the sample size was determined for overall diagnostic performance based on the area (A_z) under the composite receiver operating characteristic (ROC) curve using pooled data of all observers. We expected that the pooled A_z for the diagnosis of high-risk varices on CT is 0.95, with a width of the 95% confidence interval (CI) of 0.05. When the ratio of the number of patients with high-risk varices ranges from 1:1 to 1:6 and the ratio of standard deviation of pooled A_z with high-risk varices ranges from 1:1 to 3:1, the standard error of estimates of A_z in 100 subjects is $\leqslant$ 0.025. Thus, the 95%CI of A_z is $\leqslant$ 0.05[11].

From June 2009 to December 2010, we enrolled patients with cirrhosis as well as HCCs treated with transcatheter arterial chemoembolization (TACE), radiofrequency (RF) ablation or both procedures who were scheduled to undergo both a regular follow-up liver CT examination and a screening upper endoscopy. We excluded patients who had a history of undergoing EVL for esophageal varices[5]. One-hundred patients who agreed to participate in our prospective study underwent an endoscopy and a subsequent liver MDCT examination within four hours after endoscopy. All patients had undergone one or more regular (all within a 6-mo interval) follow-up liver MDCT examinations following TACE (n = 43), RF ablation (n = 35) or both procedures (n = 22) for HCCs. The patient population consisted of 79 men and 21 women (age range, 35-82 years; median age, 59 years, mean age, 58.4 years). Patients were diagnosed with cirrhosis based on liver histopathology (n = 28), or characteristic clinical features (symptoms and stigmata of cirrhosis and its complications), laboratory results (levels of viral markers, hyperbilirubinemia, hypoalbuminemia and coagulopathy), or imaging findings (liver configuration, splenomegaly, ascites and collateral vessels) (n = 72). Eighteen patients received β-blocker medication. All patients had liver cirrhosis due to hepatitis B infection (n = 76), hepatitis C infection (n = 14), alcohol use (n = 5) or an unknown etiology (n = 5). There were 89 patients with Child-Pugh class A, ten patients with Child-Pugh class B, and one patient with Child-Pugh class C disease.

Upper endoscopy

Patients underwent standard upper endoscopy performed by experienced gastroenterologists (with experience of performing more than 500 endoscopy examinations) in our endoscopy unit. Seven gastroenterologists performed endoscopy of the 100 patients in our study. All endoscopic studies were captured as digital imaging files. On endoscopy, esophageal varices were graded according to criteria proposed by the Japanese Research Society for Portal Hypertension[12]. Grade 2 (varices showing a beaded appearance) or grade 3 (varices running in an oblique course and showing tortuosity with a tumorfactive appearance) was chosen as a cutoff point to define high-risk varices, based on the possibility to develop esophageal bleeding[12]. Two specialized endoscopists (Joon Hyeok Lee and Moon Seok Choi) reviewed all of the stored endoscopic images and the procedure reports for all patients. The endoscopists determined the final grades of the esophageal varices of the study population based on consensus. Disagreement between the two endoscopists was resolved in conference along with a third endoscopist (Geum-Youn Gwak).

Liver computed tomography protocol

Liver CT examinations for follow-up were performed with a 64-slice MDCT scanner (Lightspeed VCT; GE Healthcare, Milwaukee, WI, United States). A total of 120 mL of nonionic contrast material (Ultravist 300, 300 mg/mL iopromide, Schering, Berlin, Germany) was administered intravenously with an automatic injector at a rate of 3-4 mL/s. Images were obtained at 30-40 s, 70 s and 180 s after the initiation of contrast material injection, representing the hepatic arterial, portal venous and equilibrium phases, respectively. In order to determine the appropriate scanning time for hepatic arterial phase imaging, we used an automatic bolus-tracking technique with automated scan-triggering software (SmartPrep; GE Healthcare). Hepatic arterial phase scanning was started automatically 12 s after the trigger threshold (100 HU) was reached at the level of the supraceliac abdominal aorta. Images were obtained from the dome of the diaphragm to the bottom of the liver during a single breath-hold. CT scanning parameters were as follows: 64 detector rows; beam collimation, 0.625 mm × 64 mm; pitch, 0.984; kVp/mA, 120/100-300; gantry rotation time, 0.6 s. Isotropic raw data were acquired with a slice thickness of 1.25 mm and an interval of 1.25 mm with the use of MDCT. Using the raw data, a transverse image was obtained with a slice thickness of 5 mm and an interval of 2.5 mm. During the portal venous phase, coronal and sagittal MPR images were obtained with a 3 mm interval and a slice thickness of 3 mm. No patients received oral contrast material.

Liver computed tomography image analysis

Three abdominal radiologists (Soon Jin Lee, Dongil Choi and Hangi Jo) independently interpreted the liver CT images in order to detect the presence of high-risk (grade 2 or 3) esophageal varices. The radiologists had 13, nine and five years experience, respectively, in the interpretation of abdominal CT images. The radiologists initially evaluated transverse images alone in a random order on a picture archiving and communication system (PACS) monitor. After three weeks, three orthogonal MPR images were assessed in another random order. The radiologists were blinded to the patient physical findings, laboratory values, previous imaging results and endoscopy results.

The CT images were reviewed to detect the presence of high-risk esophageal varices with a 4-point confidence scale for the development of variceal bleeding based on the endoscopic findings that were correlated with variceal bleeding: (1) definitely low-risk or no varices; (2) probably low-risk; (3) probably high-risk; and (4) definitely high-risk. The observers investigated beaded and tortuous varices (distinct enhancing nodular or linear lesions that abutted the surface of the esophageal lumen or protruded into the luminal space) on stack PACS images of CT scans. In addition, the observers measured the shortest diameter of the largest enhancing lesion (varix) during the portal venous phase by the use of an electronic caliper on one-format PACS images. A confidence score of 4 could be assigned if the diameter was definitely greater than 3 mm. A score of 3 was assigned if the diameter was between 2 mm to 3 mm. A score of 2 was assigned if the diameter was between 1 mm to 2 mm. A score of 1 was assigned if the diameter was less than 1 mm[4].

Statistical analysis

The results of liver CT imaging analyzed by three observers were correlated with the endoscopic grading of esophageal varices by the study coordinator (Hyojin Kim). Observer performance for the detection of high-risk esophageal varices was assessed by the use of ROC curve analysis with transverse images alone and with three orthogonal MPR images. A_z under each ROC curve by application of the maximum-likelihood curve-fitting algorithm was used to indicate the diagnostic performance for the detection of high-risk esophageal varices on liver CT images. A_z values were compared by using pseudovalues with the jackknife method developed by Dorfman *et al*[13]. Composite ROC curves for the performance of all observers were obtained by using the pooled data of the three observers.

The sensitivity and specificity for the CT diagnosis of high-risk esophageal varices were calculated. The sensitivity for the detection of varices was determined by use of the number of patients with varices assigned a score of 3 or 4. Sensitivities and specificities for transverse images alone and three orthogonal MPR images were compared with use of the McNemar test. The positive predicative value (PPV) and negative predicative value (NPV) were also calculated and compared with use of the χ^2 test and Fisher's exact test, respectively. A P value of less than 0.05 was considered as significant.

Interobserver agreements for the detection of esophageal varices and high-risk varices were evaluated using κ statistics. The degree of agreement was categorized as follows: κ values of 0.00-0.20 were considered to indicate poor agreement; κ values of 0.21-0.40, fair agreement; κ values of 0.41-0.60, moderate agreement; κ values of 0.61-0.80, good agreement; κ values of 0.81-1.00, excellent agreement[14]. Statistical analyses were performed using commercially available software packages (PASW Statistics, release 17.0.2; SPSS, Chicago, IL, United States; MedCalc, version 7.1; MedCalc Software, Mariakerke, Belgium).

RESULTS

In 50 patients, there were no esophageal varices (grade 0) as determined by endoscopy. Twenty-three patients were classified with grade 1 esophageal varices, 24 patients were classified with grade 2 esophageal varices (Figure 1), and three patients were classified with grade 3 esophageal varices. No patients had a significant endoscopic abnormality other than esophageal varices, such as an ulcer,

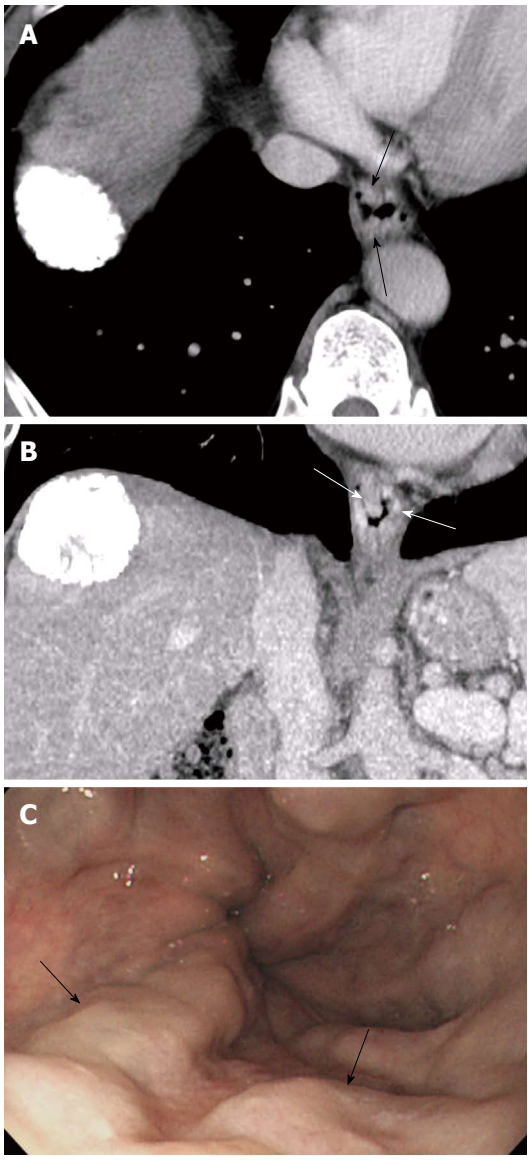


Figure 1 Imaging findings are shown for grade 2 esophageal varices in the distal esophagus in a 61-year-old man with hepatocellular carcinoma and cirrhosis. A: A transversecomputed tomography scan shows distinct enhancing nodular lesions (arrows) protruding into the luminal space. All observers recorded the lesions as high-risk esophageal varices. Periesophageal varices are not found; B: On coronal images, distinct enhancing nodular lesions (arrows) are noted, and all observers considered the lesions as high-risk esophageal varices; C: A conventional endoscopic image confirms the presence of beaded esophageal varices (arrows).

inflammation or mass. The red sign was observed in one (4.3%) of 23 patients with a grade 1 varix, 15 (62.5%) of 24 patients with a grade 2 varix and two (66.7%) of three patients with a grade 3 varix.

The A_z values of the ROC assessment of the three observers for the detection of high-risk esophageal varices with transverse images alone were 0.947 ± 0.031 (95%CI, 0.883-0.982), 0.969 ± 0.024 (95%CI, 0.913-0.993) and 0.916 ± 0.038 (95%CI, 0.843-0.962), respectively. The Az values of the ROC assessment of the three observers with the three orthogonal MPR images were 0.965 ± 0.025 (95%CI, 0.908-0.991), 0.959 ± 0.027 (95%CI, 0.899-0.988) and 0.938 ± 0.033 (95%CI, 0.871-0.976), respectively. The Az values of each observer were similar for transverse images alone and for the three orthogonal MPR images (each $P > 0.05$) (Table 1). In addition, there was no significant difference of mean Az values of pooled data by the three observer for transverse images alone (0.946 ± 0.018; 95%CI, 0.914-0.968) and for the three orthogonal MPR images (0.954 ± 0.017; 95%CI, 0.924-0.975, $P > 0.05$). The A_z values were also similar among the three observers both for transverse images alone and for the three orthogonal MPR images, respectively (each $P > 0.05$) (Table 1).

The mean sensitivity for the detection of high-risk esophageal varices of the three observers with transverse images alone and with the three orthogonal MPR images were 90.1% and 91.4%, respectively ($P > 0.05$). For each observer, the sensitivities of detecting high-risk esophageal varices were also similar for transverse images alone and for the three orthogonal MPR images (each $P > 0.05$) (Table 2). While the specificities of each observer were similar for transverse images alone and for the three orthogonal MPR images (each $P > 0.05$), the mean specificity with the three orthogonal MPR images (89.5%) was better than that with transverse images alone (86.3%) ($P = 0.039$) (Table 2).

There was no significant difference for the mean PPV of three observers for transverse images alone (70.9%) and for the three orthogonal MPR images (76.3%, $P > 0.05$). For each observer, the PPVs were also similar for transverse images alone and for the three orthogonal MPR images (each $P > 0.05$) (Table 3). There was no significant difference of the mean NPV for transverse images alone (95.9%) and for the three orthogonal MPR images (96.6%, $P > 0.05$). For each observer, the NPVs were also similar for transverse images alone and for the three orthogonal MPR images (each $P > 0.05$) (Table 3).

All κ values for agreements among the three observers with transverse images alone showed excellent agreements (range: 0.845-0.933), and κ values for agreements among three observers with three orthogonal MPR images demonstrated good and excellent agreements (range: 0.791-0.910).

DISCUSSION

Although liver CT images always cover the distal esophagus where all varices occur, some radiologists do not comment on either the presence or the degree of esophageal varices in radiological reports. Some endoscopists are unaware that liver CT scans provide excellent images of esophageal varices. Regular follow-up CT examinations are essential to evaluate recurrence and therapeutic effectiveness in patients treated with TACE or ablation therapy for HCC[10,15]. In such patients, we can make an objective decision about the risk of variceal bleeding with the use of regular follow-up CT imaging without the use of an invasive endoscopic evaluation. The presence of periesophageal varices has been correlated with recur-

Table 1 Az in receiver operating characteristic curve analysis and *P* values of liver computed tomography for the detection of high-risk esophageal varices by three observers

	Observer 1 Transverse	Observer 1 3 MPR	Observer 2 Transverse	Observer 2 3 MPR	Observer 3 Transverse	Observer 3 3 MPR	Mean Transverse	Mean 3 MPR
ROC Az	0.947 ± 0.031	0.965 ± 0.025	0.969 ± 0.024	0.959 ± 0.027	0.916 ± 0.038	0.938 ± 0.033	0.946 ± 0.018	0.954 ± 0.017
95%CI	0.883-0.982	0.908-0.991	0.913-0.993	0.899-0.988	0.843-0.962	0.871-0.976	0.914-0.968	0.924-0.975
P value[1]	0.318		0.328		0.271		0.367	
P value[2]	Observer 1-2 0.368	Observer 1-2 0.782	Observer 2-3 0.099	Observer 2-3 0.461	Observer 3-1 0.35	Observer 3-1 0.334		

[1]Represent the differences of the A_z values for transverse images alone and for the three orthogonal MPR images by each observer and the pooled data; [2]Represent the differences (interobserver comparisons) of the Az values among three observers with both transverse images alone and with the three orthogonal MPR images.Transverse indicates transverse images alone and 3 MPR indicates three orthogonal MPR images. Az values are mean ± SD. CI: Confidence interval; MPR: Multiplanar reformation; ROC: Receiver operating characteristic.

Table 2 Sensitivity and specificity of liver computed tomography for the detection of high-risk esophageal varices by three observers

	Observer 1 Transverse	Observer 1 3 MPR	Observer 2 Transverse	Observer 2 3 MPR	Observer 3 Transverse	Observer 3 3 MPR	Mean Transverse	Mean 3 MPR
Sensitivity%	92.6 (25/27)	92.6 (25/27)	92.6 (25/27)	92.6 (25/27)	85.2 (23/27)	88.9 (24/27)	90.1 (73/81)	91.4 (74/81)
P value	1.000		1.000		1.000		1.000	
Specificity%	89.0 (65/73)	90.4 (66/73)	87.7 (64/73)	87.7 (64/73)	82.2 (60/73)	90.4 (66/73)	86.3 (189/219)	89.5 (196/219)
P value	1.000		1.000		0.070		0.039	

For sensitivity, the numbers in parentheses are the number of varices assigned a score of 3 or 4 divided by the total number of varices. *P* values represent the differences of the sensitivity and the specificity for transverse images alone and for the three orthogonal MPR images (McNemar test). MPR: Multiplanar reformation.

Table 3 Positive predictive value and negative predictive value of liver computed tomography for the detection of high-risk esophageal varices by three observers

	Observer 1 Transverse	Observer 1 3 MPR	Observer 2 Transverse	Observer 2 3 MPR	Observer 3 Transverse	Observer 3 3 MPR	All Transverse	All 3 MPR
PPV %	75.8 (25/33)	78.1 (25/32)	73.5 (25/34)	73.5 (25/34)	63.9 (23/36)	77.4 (24/31)	70.9 (73/103)	76.3 (74/97)
P value	0.947		1.000		0.352		0.482	
NPV%	97.0 (65/67)	97.1 (66/68)	97.0 (64/66)	97.0 (64/66)	93.8 (60/64)	95.7 (66/69)	95.9 (189/197)	96.6 (196/203)
P value	1.000		1.000		0.710		0.797	

P values represent the differences of the positive predicative value and the negative predicative value for transverse images alone and for the three orthogonal MPR images (χ^2 test and Fisher's exact test). MPR: Multiplanar reformation; PPV: Positive predicative value; NPV: Negative predicative value.

rence of luminal esophageal varices after treatment[16].

As with the results of previous reports[4-8], the sensitivities for the detection of high-risk esophageal varices for each observer were also high (approximately 90%) in our study (Table 2). Excellent NPVs as well as high sensitivities for the detection of high-risk varices are clinically important, as the goal of endoscopic screening for esophageal varices is to decrease morbidity and mortality resulting from variceal bleeding. As good screening tests should show a low frequency of false negative results, sensitivity and NPV are more important as compared to specificity and PPV in a screening test[17]. Even though both PPV and NPV are influenced by disease prevalence, the prevalence of high-risk esophageal varices in our study was similar to prior studies[4-8].

A recent study[7] mentioned the possibility that sagittal and coronal images with isotropic spacial resolution potentially decrease measurement variability as vessels are displayed within the imaging plane. However, based on the results of our study, additional coronal and sagittal images showed little practical help to evaluate high-risk esophageal varices when compared to assessment of transverse images alone. Moreover, the use of virtual esophagography proposed in another recent study[5] might be impractical because of time-consuming and invasive insertion of the esophagus with a catheter for air insufflation. Insufflation alters both the luminal pressure and the hemodynamics of the varices. Perri *et al*[7] pointed out that the measurement of varices in a non-distended state might provide a more proper assessment for bleeding risk as variceal bleeding occurs while the esophagus is in its normal non-distended state.

Our study has some limitations. The most important limitation is the use of endoscopy as the reference stan-

dard. Prior studies[18,19] have demonstrated a lack of good correlation between endoscopists to evaluate the grades of esophageal varices. In our study, given the semi-open access system of the endoscopy unit, many different endoscopists perform endoscopy procedures. Hence, specialized endoscopists re-evaluated the grades of esophageal varices with review of the stored endoscopic images. Second, we could not evaluate qualitative endoscopic appearances including the presence of red signs such as cherry-red spots and diffuse redness, which is one of the predictors of variceal bleeding. These red signs cannot be depicted on CT images. In a recent report[20], cases with the shortest diameter of the largest varix of more than 4 mm showed positive red signs. In our study, the endoscopic red sign was observed in only one patient with low-risk (grade 1) esophageal varices (4.3%, 1 of 23 cases)[4].

In conclusion, our results have demonstrated excellent diagnostic performance, sensitivity and NPV with low interobserver variability to detect high-risk esophageal varices on regular follow-up liver MDCT examinations in patients treated with locoregional therapy for HCCs. Careful evaluation of high-risk esophageal varices on a liver MDCT examination may be useful to avoid performing low-yield endoscopy in such patients. When compared to the assessment of transverse images alone, additional coronal and sagittal images were of little help to evaluate high-risk esophageal varices. Our results suggest that liver CT can be used as a single noninvasive surveillance tool for both esophageal varices and recurrent HCC after locoregional therapy. However, a definite change of clinical pathways may be needed in a randomized controlled study on the comparison of bleeding frequency and survival in patients treated with locoregional therapy between the use of a regular follow-up liver MDCT examination alone and with the combination of screening endoscopy.

COMMENTS

Background

The presence of high-risk varices seen in up to 30% of patients with esophageal varices on endoscopy is considered as an indication for prophylaxis against variceal bleeding with either endoscopic variceal ligation or the administration of β-blockers. Several recent studies have suggested the usefulness of computed tomography (CT) scans to evaluate esophageal varices, and one study asserted that CT was a better initial screening modality when compared to endoscopy in terms of cost-effectiveness and patient preference.

Research frontiers

As CT is commonly used as a follow-up imaging modality in patients treated with locoregional therapy for hepatocellular carcinoma (HCC), the authors suggested that replacement of CT for evaluating varices was more persuasive in such patients. Careful evaluation of high-risk esophageal varices on a liver multi-detector row computed tomography (MDCT) examination may be useful to avoid performing low-yield endoscopy in such patients.

Innovations and breakthroughs

The results of this study have demonstrated excellent diagnostic performance, sensitivity and negative predicative value with low interobserver variability to detect high-risk esophageal varices on regular follow-up liver MDCT examinations in patients treated with locoregional therapy for HCCs. When compared to the assessment of transverse images alone, additional coronal and sagittal images were of little help to evaluate high-risk esophageal varices.

Applications

The study results suggest that that liver CT can be used as a single noninvasive surveillance tool for both esophageal varices and recurrent HCC after locoregional therapy.

Peer review

This is a good prospective study in which authors evaluate the diagnostic performance of radiologists for the detection of high-risk esophageal varices on regular follow-up liver multi-detector row CT examinations in patients treated with locoregional therapy for HCC.

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BRIEF ARTICLE

Pentoxifylline in hepatopulmonary syndrome

Hamid Reza Kianifar, Maryam Khalesi, Eftekhar Mahmoodi, Monavar Afzal Aghaei

Hamid Reza Kianifar, Allergy Research Center, Ghaem Medical Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad 91735, Iran
Maryam Khalesi, Department of Pediatrics, Ghaem Medical Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad 91735, Iran
Eftekhar Mahmoodi, Department of Pediatric Cardiology, Ghaem Medical Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad 91735, Iran
Monavar Afzal Aghaei, Department of Biostatistics, Faculty of Health, Mashhad University of Medical Sciences, Mashhad 91735, Iran
Author contributions: Kianifar HR is the primary author that contributed to the intellectual content, designed the research, drafted the article, and performed the literature research; Khalesi M is the secondary author who contributed equally to the first author in research performance; Mahmoodi E contributed to this study by echocardiography and data collection; Afzal Aghaei M contributed to data collection and analyzed the data.
Supported by Research Council of Mashhad University of Medical Sciences
Correspondence to: Hamid Reza Kianifar, MD, Associate Professor of Pediatric Gastroenterology, Allergy Research Center, Ghaem Medical Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad 91735, Iran. kianifarhr@mums.ac.ir
Telephone: +98-511-8012762 Fax: +98-511-8458769
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Abstract

AIM: To determine the effects of pentoxifylline (PTX) on clinical manifestations and evaluate arterial blood gas data in hepatopulmonary syndrome (HPS) in children.

METHODS: In a pilot study of 10 children with chronic liver disease, who had HPS, 20 mg/kg/d PTX was administered for 3 mo. Clinical data and arterial blood gas parameters were evaluated at baseline, the end of the treatment period, and 3 mo after drug discontinuation.

RESULTS: Six patients could tolerate PTX, while four patients experienced complications. Among patients who could tolerate PTX, there was a significant increase in arterial oxygen pressure (PaO_2) (P = 0.02) and oxygen saturation (SaO_2) (P = 0.04) and alveolar-arterial oxygen gradient (P = 0.02) after 3 mo of treatment. Significant decreases in PaO_2 (P = 0.02) and alveolar-arterial oxygen gradient (P = 0.02) were also seen after drug discontinuation.

CONCLUSION: PTX may improve PaO_2, SaO_2 and alveolar-arterial oxygen gradient in the early stage of HPS.

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Key words: Pentoxifylline; Hepatopulmonary syndrome; Child; Arterial oxygen pressure; Therapy; Alveolar-arterial oxygen gradient

Peer reviewers: Sabahattin Kaymakoglu, Professor, Department of Gastroenterohepatology, Istanbul Medical Faculty, Capa, 34390 Istanbul, Turkey; Dr. Assy Nimer, MD, Assistant Professor, Liver Unit, Ziv Medical Centre, BOX 1008, Safed 13100, Israel

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INTRODUCTION

The hepatopulmonary syndrome (HPS) is the triad of liver disease, arterial hypoxemia and widespread pulmonary vascular dilatation[1-3]. Prevalence of this syndrome is 10%-30% among patients with chronic liver disease[4].

The pathogenesis of HPS is unclear. The roles of eicosanoids as vasoconstrictors and the increased number of intravascular macrophage-like cells have been reported previously. Increased pulmonary production of NO in

cirrhosis has been considered as a reason for intrapulmonary venous dilatation. It is claimed that increased pulmonary NO production is due to raised pulmonary vascular endothelial NO synthase (eNOS) and inducible NO synthase expression. Recent studies have shown that increased hepatic production and release of low level of endothelin 1 (ET-1) can act as a trigger for enhancing eNO level. Tumor necrosis factor α (TNFα) and ET-1 have been implicated in the development of experimental HPS. Recent studies have suggested that progression of HPS is due to increased CO production and heme oxygenase 1 expression[5-7]. The presence of HPS increases mortality in cirrhotic patients and may influence the frequency and severity of complications of portal hypertension[8-11]. To the best of our knowledge, there is currently no effective medical therapy for HPS. Several medications such as methylene blue, garlic and aspirin have been reported to have a therapeutic effect[12-15], but curative treatment of this syndrome is still orthotopic liver transplantation[16].

Pentoxifylline (PTX), a nonspecific phosphodiesterase-4 inhibitor, blocks TNFα synthesis[17,18] and seems to prevent HPS. There are limited studies about PTX in adult patients with HPS, with varying results. Until now, no study has been carried out in children. The aim of this study was to evaluate the effect of PTX on clinical manifestations and arterial blood gas data in children with HPS.

MATERIALS AND METHODS

This pilot study was carried out in the Ghaem Medical Center of Mashhad University of Medical Sciences, Iran during a 21-mo period (March 2008-November 2010). The protocol for this study was approved by the Ethics Committee of Mashhad University. Children and their parents were given informed consent forms. A consecutive series of patients with cirrhosis attending our hospital were screened for HPS. HPS was diagnosed according to the European Respiratory Society Task Force criteria: hypoxemia plus positive contrast-enhanced echocardiography[7]. Positive contrast echocardiography was defined by the appearance of air bubbles in the left side of the heart after the first three beats during injection of 10 mL agitated isotonic saline solution in the antecubital vein[19]. Exclusion criteria were active infection, known malignancy, cardiac disease, and kidney disease.

Patient data including demographics, etiology of cirrhosis, clinical manifestations, laboratory results [complete blood count (CBC), diff, blood urea nitrogen, creatinine, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, prothrombin time, partial thromboplastin time, total bilirubin, direct bilirubin, albumin and total protein] were recorded. For each patient, Child score was determined. All patients underwent measurement of arterial oxygen pressure (PaO_2) and oxygen saturation (SaO_2) by blood sampling from the radial artery in a sitting position while breathing ambient air. For each patient, alveolar-arterial oxygen gradient was calculated. Patients were classified in four groups according to severity of hypoxemia: mild HPS with $PaO_2 \geqslant 80$ mmHg, moderate HPS with $60 \leqslant PaO_2 < 80$ mmHg, severe HPS with $50 \leqslant PaO_2 < 60$ mmHg, and very severe HPS with $PaO_2 < 50$ mmHg[7].

Table 1 Baseline patient characteristics *n* (%)

Variable	Value (*n* = 10)
Age (yr)	
Mean ± SD, (range)	9.2 ± 5.0 (2-16)
Sex	
Female/male	4/6
Cause of cirrhosis	
Wilson disease	3 (30)
Cryptogenic cirrhosis	3 (30)
Autoimmune hepatitis	2 (20)
PFIC	1 (10)
GSD	1 (10)
Staging of HPS	
Mild	2 (20)
Moderate	6 (60)
Severe	1 (10)
Very severe	1 (10)
Child score	
A	3 (30)
B	4 (40)
C	3 (30)

PFIC: Progressive familial intrahepatic cholestasis; GSD: Glycogen storage disease; HPS: Hepatopulmonary syndrome.

PTX at a dose of 20 mg/kg per day orally in three divided doses was administrated for 3 mo. In this period, patients were followed up weekly and whenever patients required, by telephone conference and monthly with outpatient visits. Clinical manifestations and laboratory data were rechecked at the end of the treatment period, 3 mo after drug discontinuation and whenever a child had any deterioration or could not tolerate PTX. Response to PTX was considered as increased $PaO_2 > 10$ mmHg from baseline[20]. Complement a three months period of treatment was considered as primary end point and development of serious side effects, death and liver transplantation was considered as the secondary end point.

After entering data into SPSS version 16.0 software, quantitative data were analyzed by the Wilcoxon signed rank test and qualitative data by the McNemar test. A P value < 0.05 was considered significant.

RESULTS

A total of 65 patients aged < 18 years with cirrhosis were screened for the presence of HPS from March to August 2009. Twelve patients had HPS. Two were excluded. One had kidney disease and one did not allow us to intervene. Ten children were enrolled. Two patients had mild HPS, six moderate HPS, one severe HPS and one very severe HPS. Four patients were girls. Mean age was 9.2 ± 5.0 years old. The most common recognized cause of cirrhosis was Wilson's disease (Table 1). Minimum alveolar-

Table 2 Clinical and laboratory data at baseline and end of treatment period (mean ± SD) *n* (%)

Variable	Baseline	End of treatment	*P* value
Clinical manifestations			
Cyanosis	9 (90)	5 (50)	0.12
Dyspnea	3 (30)	2 (20)	1.0
Clubbing	7 (70)	7 (70)	1.0
Palmar erythema	5 (50)	5 (50)	1.0
Hepatomegaly	6 (60)	6 (60)	1.0
Splenomegaly	7 (70)	7 (70)	1.0
Laboratory findings			
White blood cell count, $\times 10^3$/mm	6.4 ± 4.41	4.36 ± 2.04	0.17
Hemoglobin, g/dL	11.25 ± 2.30	11.45 ± 2.71	0.49
Platelet count, $\times 10^3$/mm^3	131.6 ± 566.74	156.2 ± 498.36	0.32
Serum creatinine, mg/dL	0.56 ± 0.16	0.6 ± 0.14	1.0
Serum urea, g/dL	12.60 ± 5.98	14.70 ± 7.20	0.34
Serum albumin, g/dL	3.88 ± 0.52	3.56 ± 0.15	0.59
Serum total protein, g/dL	7.20 ± 0.40	7.26 ± 0.81	1.0
PT, s	15.72 ± 3.07	18.51 ± 8..27	0.40
PTT, s	45.50 ± 14.54	49.42 ± 11.41	0.24
INR	1.46 ± 0.36	1.90 ± 1.14	0.67
Total bilirubin, mg/dL	5.73 ± 7.39	5.07 ± 6.04	0.17
Direct bilirubin, mg/dL	1.70 ± 2.50	1.68 ± 2.71	0.83
ALT, U/L	83.60 ± 64.28	40.60 ± 31.56	0.27
AST, U/L	92.55 ± 64.48	66.80 ± 2814	0.50
ALP, U/L	564.66 ± 198.74	536.50 ± 17.67	0.18

PT: Prothrombin time; PTT: Partial thromboplastin time; INR: International normalized ratio; ALT: Alaninaminotranferase; AST: Aspartataminotransferase; ALP: Alkaline phosphatase.

arterial oxygen gradient at baseline was 18.84 and maximum was 71.98. Clinical and laboratory characteristics of the patients at baseline and end of treatment period are summarized in Table 2.

Six patients completed the treatment period. Petechiae with platelet count 10 000/mm^3 was seen in one patient, 3 wk after the beginning of PTX. He was a 16-year-old boy with Wilson's disease and very severe HPS. His platelet count at the beginning of the study was 159 000/mm^3. Platelet count increased to 100 000/mm^3 2 wk after drug discontinuation. Other laboratory tests did not show any significant change. Although nausea was seen in all patients, only three had severe vomiting that did not respond to symptomatic treatment and caused drug withdrawal. Among them, two had moderate HPS and one had severe HPS. No significant laboratory changes were seen in these patients. Treatment periods were 10 d, 28 d and 45 d. Other adverse effects did not cause drug withdrawal and improved with symptomatic treatment (two patients with fatigue and two with headache).

Among patients who completed the treatment period, average increment in PaO_2 was 26 mmHg and SaO_2 was 4.3%. At least 10 mmHg elevations in PaO_2 level was seen in all patients. Also, PaO_2 significantly decreased 3 mo after the end of treatment. Moreover, there was significant improvement in alveolar-arterial oxygen gradient at the end of the treatment period and a significant increase after drug discontinuation (Table 3). Among these patients, two had mild HPS and four had moderate HPS.

For clinical manifestations, there were no significant change between baseline, the end of treatment, and 3 mo later. There was also no significant change in other laboratory tests. In a 12-mo period of follow-up after drug discontinuation, two patients died. The first was the 16-year-old boy with Wilson's disease. He died from upper gastroesophageal bleeding from esophageal varicose veins, 8 mo after drug withdrawal. The second was a girl of 2 years with cryptogenic cirrhosis who received PTX for 10 d. She was one of the three patients who could not tolerate PTX because of severe vomiting. She died due to hepatic encephalopathy and septicemia.

Table 3 PaO_2, SaO_2 and alveolar-arterial oxygen gradient (A-a G) before, at the end and 3 mo after treatment among patients who could tolerate pentoxifylline (mean ± SD)

Variables	PaO_2	SaO_2	A-a G
Baseline	73.3 ± 9.33	93.33 ± 6.28	40.10 ± 18.09
End of treatment	97.0 ± 3.79	97.66 ± 1.50	20.73 ± 9.46
Three months after treatment	71.83 ± 21.47	90.5 ± 8.6	43.68 ± 24.30
P value	0.02[1], 0.02[2]	0.04[1], 0.05[2]	0.02[1], 0.02[2]

[1]Baseline *vs* end of treatment; [2]End of treatment *vs* 3 mo later. PaO_2: Oxygen pressure; SaO_2: Oxygen saturation.

DISCUSSION

In this pilot study, we found significant improvements in PaO_2, SaO_2 and alveolar-arterial oxygen gradient after 3 mo therapy among patients who could tolerate PTX. PaO_2 was also significantly decreased at 3 mo after drug withdrawal, which supports the therapeutic effects of PTX. However, we did not find any significant change in clinical parameters. In this study, 40% of patients could not complete the treatment period. In other studies of patients with liver disease, drug withdrawal rate due to adverse effects ranged from 0% to 40%[17,21,22]. It seems that gastrointestinal intolerance was the most common cause (nausea in 100% and vomiting in 30%). Previously, animal studies have shown that inhibition of TNFα with PTX in cirrhotic rats after common bile duct ligation can improve HPS[23,24].

Tanikella *et al*[25] selected nine adult patients with moderate to severe HPS. There was no significant difference in PaO_2 and alveolar-arterial oxygen gradient before and after PTX administration. High incidences of gastrointestinal adverse effects were the most important reason for intolerance. This resulted in a 44% attrition rate and only one patient (12%) could tolerate PTX for the complete treatment period of 8 wk.

Despite these results, Gupta *et al*[26] have demonstrated that 3 mo treatment with PTX can improve the symptoms and signs in about 90% of patients. There was also a significant increase in median supine, standing and postexercise PaO_2 ($P < 0.01$). Side effects were reported in a few patients (vomiting in three and dyspnea in two), but they did not require drug withdrawal.

In our study, among the four patients who could not

tolerate PTX, two had moderate, one had severe and one had very severe HPS. Also, three patients had Child score C and one had Child score B. The high incidence of intolerance in these patients was comparable with that of the Tanikella *et al*[25] study that was carried out in patients with moderate to severe HPS, and suggests that beginning PTX in the early stages of HPS may increase tolerability and effectiveness.

As far as we are aware, no study has determined the optimal dose of PTX in cirrhotic children. In animal studies, the dose of PTX used was 10-50 mg/kg per day[4,23,24]. PTX was used previously in children for treatment of Kawasaki disease as an antiplatelet drug with the same dose and no side effects were seen[27]. One potential explanation for this difference is significant first pass metabolism of PTX in the liver to an active metabolite, and consequently, serum levels are significantly high in liver dysfunction[28]. This problem does not occur in Kawasaki disease. Furthermore, finding the optimal dosage of PTX in liver dysfunction can be beneficial. In the present study, one patient experienced thrombocytopenia. Although thrombocytopenia is a manifestation of cirrhosis, decreased platelet count after administration of PTX, and increased platelet count after drug withdrawal denote the role of PTX. This implies that CBC is an important laboratory test during PTX administration. We did not observe any change in serum creatinine or liver function before and after therapy with pentoxifylline that may have contributed to changes in pentoxifylline metabolism.

To evaluate effects of PTX in HPS, we suggest double-blind studies, long-term treatment, and contrast echocardiography at the end of the treatment period for later studies. Meanwhile, it is necessary to evaluate optimal dose of PTX in cirrhotic children. In conclusion, it seems that PTX may have beneficial effects on oxygenation in early stages of HPS.

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COMMENTS

Background

Hepatopulmonary syndrome (HPS) is a triad of liver disease, arterial hypoxemia and widespread pulmonary vascular dilatation. There is currently no effective medical therapy for HPS in spite of several recommended medications. Recently, pentoxifylline (PTX), a nonspecific phosphodiesterase-4 inhibitor, which blocks tumor necrosis factor α (TNFα) synthesis, has appeared to prevent HPS. The aim of this study was to evaluate the effect of PTX on clinical manifestations and arterial blood gas data in children with HPS.

Research frontiers

This study, possibly for the first time, reported therapeutic effects of PTX in children with HPS. The authors concluded that there were no significant differences in clinical manifestations and other laboratory tests after treatment. PTX may improve arterial oxygen tension, oxygen saturation and alveolar-arterial oxygen gradient in the early stage of HPS. Thus, it seems that PTX may have beneficial effects on oxygenation in early stages of HPS.

Innovations and breakthroughs

PTX, a nonspecific phosphodiesterase-4 inhibitor, blocks TNFα synthesis and seems to prevent HPS. The authors evaluated, perhaps for the first time, the therapeutic efficacy of PTX in children with HPS.

Applications

The authors concluded that PTX may have beneficial effects on oxygenation in early stages of HPS. However, the authors suggest further double blind studies with longer treatment periods and contrast echocardiography at the end of the treatment period until the effect of PTX in HPS are defined clearly. Also, the authors recommend evaluation of the optimal dose of PTX in cirrhotic children.

Peer review

This is a prospective study with a few patients. It is accepted with minor revisions.

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BRIEF ARTICLE

Inducible nitric oxide synthetase genotype and *Helicobacter pylori* infection affect gastric cancer risk

Alireza Rafiei, Vahid Hosseini, Ghasem Janbabai, Bahman Fazli, Abulghasem Ajami, Zahra Hosseini-khah, Jeremy J Gilbreath, D Scott Merrell

Alireza Rafiei, Bahman Fazli, Abulghasem Ajami, Zahra Hosseini-khah, Faculty of Medicine, Molecular and Cell Biology Research Center, Mazandaran University of Medical Sciences, Sari 48175-1665, Iran
Vahid Hosseini, Department of Internal Medicine, Mazandaran University of Medical Sciences, Sari 48175-1665, Iran
Ghasem Janbabai, Department of Internal Medicine, Imam Hospital, Mazandaran University of Medical Sciences, Sari 48175-1665, Iran
Jeremy J Gilbreath, D Scott Merrell, Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, United States
Author contributions: Rafiei A and Fazli B designed the study protocol; Hosseini V performed endoscopy and evaluated case findings; Janbabai G performed histopathological examination and staging of the patients; Ajami A and Fazli B performed serology tests; Hosseini-khah Z and Fazli B performed molecular tests and screened patients; Rafiei A and Ajami A did the statistical analysis; Rafiei A, Gilbreath J and Merrell DS analyzed data and wrote the manuscript.
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Correspondence to: Dr. Bahman Fazli, Faculty of Medicine, Molecular and Cell Biology Research Center, Mazandaran University of Medical Sciences, 18KM Khazar Blvd, Khazar Sq., Sari 48175-1665, Iran. falib100@gmail.com
Telephone: +98-151-3543088 Fax: +98-151-3543087
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Abstract

AIM: To investigate the association of the inducible nitric oxide synthetase (*iNOS*) C150T polymorphism with *Helicobacter pylori* (*H. pylori*) infection and gastric cancer (GC) risk in Iran.

METHODS: In order to determine whether there was a correlation between *iNOS* genotype and GC in Iran, we conducted a case-control study using samples from 329 individuals. For each sample, the C150T *iNOS* polymorphism was genotyped by polymerase chain reaction (PCR) and restriction digestion. Patients were grouped by cancer presence, demographic and behavior characteristics, and *H. pylori* infection status. Statistical tests were conducted to determine whether any behavioral factors or a particular *iNOS* genotype was associated with GC in the study population.

RESULTS: In this population, we found that smoking, hot beverage consumption, a familial history of GC and *H. pylori* infection status were significantly associated with GC development ($P = 0.015$, $P < 0.001$, $P = 0.0034$, and $P < 0.015$, respectively). The distribution of the C150T *iNOS* genotypes among the two study groups was not statistically significant alone, but was impacted by *H. pylori* infection status. When compared to the non-*H. pylori* infected group, cancer patients who had a heterozygous CT genotype and were also infected with *H. pylori* were 2.1 times more at risk of developing GC [odds ratio (OR) = 2.1, $P = 0.03$] while those with a homozygous TT genotype and infected with *H. pylori* were 5.0 times more at risk of developing GC (OR = 5.0, $P = 0.029$). In contrast, this association was not seen in patients in the control group.

CONCLUSION: A CT or TT polymorphism at position 150 in the *iNOS* gene significantly increases the risk of GC and may be a marker for GC susceptibility.

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Key words: Inducible nitric oxide synthetase; Gastric cancer; *Helicobacter pylori*; Heterozygous CT genotype; Homozygous TT genotype

Peer reviewers: Dr. Thomas Wex, Clinic of Gastroenterology and Hepatology, Otto-von-Guericke University, Leipziger Str. 44, 39120 Magdeburg, Germany; Dr. Francesco Franceschi, Internal Medicine, Catholic University of Rome, Policlinico A Gemelli, 00168 Rome, Italy; Guang-Wen Cao, Professor, Department of

Epidemiology, Second Military Hospital Shanghai, 800 Xiangyin Rd., Shanghai 200433, China

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INTRODUCTION

Gastric cancer (GC) is a major cause of morbidity and mortality throughout the world; it is estimated that GC is responsible for over 700 000 deaths per year[1,2], which makes this disease one of the leading causes of cancer-related death worldwide[3]. One of the most prominent risk factors for development of GC is infection with the bacterium *Helicobacter pylori* (*H. pylori*). Indeed, since its discovery in the early 1980s, *H. pylori* has been recognized as the causative agent of two types of cancer, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma[4-8]. The tight association between infection with this pathogen and the development of GC led to the classification of *H. pylori* as a class I carcinogen[9]; it is currently the only bacterium so classified.

Although *H. pylori* infection is relatively common, only a small subset of infected individuals develop GC; some individuals colonized with *H. pylori* carry the organism their entire lives with no overt disease symptoms, while others develop severe disease. Furthermore, recent evidence has begun to suggest that *H. pylori* colonization may be beneficial in some individuals; studies indicate that *H. pylori* protects against the development of esophageal cancer, childhood asthma[10-12] and active tuberculosis[10]. These findings have led researchers to try to define risk factors associated with *H. pylori*-induced disease development. Indeed, years of study have shown that in *H. pylori*-infected individuals, disease development/carcinogenesis is a complex and multifactorial process that involves an intricate interplay between numerous factors; studies indicate that disease etiology is influenced by a combination of bacterial factors, host genetics, and environmental factors such as diet.

Numerous bacterial factors have been identified as being important for *H. pylori*-induced disease development. These include the toxins CagA[13,14] and VacA[15,16], the adhesions BabA[17] and SabA[18], and the outer membrane proteins HomA, HomB, and OipA[19-21]. These and other bacterial factors act in concert to alter host cell signaling pathways, induce epithelial cell proliferation, and disrupt the delicate balance that exists between the bacterium and the host immune system. However, although these bacterial factors play a key role in the development of gastric disease, they represent only one aspect of the story. Indeed, there is a large body of work that suggests environmental factors also affect the risk of GC development. Some of the more prominent environmental risk factors include smoking[22] and high levels of dietary salt intake[23-25].

Besides these exogenous factors, the host immune system also clearly plays a major role in the development of gastric disease. In response to infection with *H. pylori*, the localized gastric environment becomes pro-inflammatory *via* the expression of pro-inflammatory cytokines[26-28]. Because these host factors play an important role in generating and maintaining the pro-inflammatory environment, it is perhaps not surprising that specific mutations in the genes that encode these factors influence gastric disease development. For example, specific interleukin-1 (*IL-1*), *IL-8*, *IL-10*, and tumor necrosis factor-α gene polymorphisms are associated with an increased risk for GC[29,30]. While these cytokines play an important role in the development and maintenance of the pro-inflammatory environment in the gastric mucosa, there are clearly other host factors involved in the disease process.

Nitric oxide (NO) is a molecule that has multiple functions in the gastrointestinal tract. In addition to having anti-microbial properties and playing an active role in vasodilation[31], NO is also a component of the pro-inflammatory immune response that has been linked to carcinogenesis[32]. NO is synthesized from L-arginine by three distinct isoforms of the nitric oxide synthetase (NOS)[31]. Whereas 2 of the 3 isoforms (neuronal NOS and epithelial NOS) are constitutively expressed, the inducible isoform (iNOS) is primarily expressed in response to specific cytokines[31]. Furthermore, expression of iNOS is upregulated during *H. pylori* infection[33]. Several studies have shown a link between increased levels of iNOS and risk of GC development[34,35]. Since one of the end products of NO metabolism is reactive nitrogen species, it is hypothesized that higher levels of iNOS lead to more oxidative DNA damage, thus increasing the risk of cancer development[31]. Interestingly, a single amino acid substitution (Ser 608 Leu) in the iNOS reductase domain has been associated with an increased risk for GC in Asian populations[36,37]. This amino acid change is the result of a single polymorphism (C150T) in exon 16[36,37], which is believed to affect the activity of the iNOS enzyme[36].

Even though the number of GC cases has decreased in many countries in recent years, the incidence of GC in Northern Iran remains high[38]. Several provinces, including Manzadaran, Ardabil, Golestan, Semnan, and the metropolitan area of Tehran, have high rates of GC in both men and women[38]. The age-standardized incidence rate for GC in these areas has been reported as high as 49.1 in men, and 25.4 in women[38]. These incidence rates may be at least partially explained by the fact that a significant proportion of this population is colonized by *H. pylori*. Indeed, the *H. pylori* infection rate for Iranians aged 40 years and over can be as high as nearly 90%[39,40]. A similar prevalence was reported for the Babol city of Iran where the *H. pylori* infection rates are 78% for men and 82% for women[41]. In addition to high rates of *H.*

pylori infection, environmental factors such as consumption of red meat and dairy products, high salt intake, and consuming hot tea have been identified as important risk factors for GC in this region[2].

At present, virtually nothing is known concerning which host genetic factors may influence GC risk in this region. Herein, we describe an epidemiologic study in which we investigate the association of the *iNOS* C150T polymorphism with *H. pylori* infection and GC risk among men and women from the Mazandaran region of Iran. We found that in this population, individuals who are infected with *H. pylori* are at a significantly higher risk for development of GC if they also harbor a CT or TT *iNOS* genotype. To date, this report is the first study that links a host-genetic factor to GC risk in this population. Furthermore, it is the first study to investigate the importance of the *iNOS* polymorphism in a non-Asian population. *En masse*, our data indicated that the *iNOS* C150 allele may be a potential marker for susceptibility to *H. pylori*-mediated GC development in multiple populations.

MATERIALS AND METHODS

Study participants

This case-control study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Medical Research Ethics Committee at the Mazandaran University of Medical Sciences. Written informed consent was obtained from each individual, and all study participants were given an explanation of the nature of the study prior to enrollment. Patients were enrolled in the study after seeking treatment at Touba Polyclinics or Imam Hospital between April 2008 and March 2011. Patients with GC were diagnosed by gastric endoscopy and cases were defined using the International Classification of Diseases for Oncology and Lauren criteria[42]. During the course of the study, 159 individuals with GC were enrolled. This group had a mean age of 61.32 ± 12.5 years and was composed of 75 female (approximately 47%) and 84 male (approximately 53%) patients. Patients with inflammatory diseases such as rheumatoid arthritis and/or malignancies other than gastric carcinoma were excluded from the study. Members of the control group were matched by age, sex, geographic location, and ethnic background. This group included 170 individuals with a mean age of 58.86 ± 14.2 years. Ninety-eight were male (approximately 58%) and 72 were female (approximately 42%). For this population, healthy controls who were 40 years of age or greater were recruited from the clinics after undergoing an annual medical screening that included a gastric endoscopy. Healthy controls who were less than 40 years of age were recruited after being referred to the clinic for routine laboratory testing. Demographic information, eating habits, and epidemiologic risk factors were provided by study participants during a face-to-face interview. Risk factors that were evaluated included cigarette smoking, drinking hot beverages, and consumption of salted fish and fast food. A person was considered a smoker if they reported smoking at least one cigarette per day for 12 or more months. Consumption of salted fish or fast food was defined as consuming either item one or more times a week for at least 6 mo. Criteria for drinking hot beverages were essentially as described previously[43]. Patients were considered to have a family history of GC if either of their parents currently had or had been diagnosed with GC in the past, or if any of their siblings had a history of the disease.

Inducible nitric oxide synthetase genotyping

In order to investigate *iNOS* gene polymorphisms, venous blood was collected from study participants and used to isolate genomic DNA by the salting-out method[44]. *iNOS* genotyping was performed using an amplicon-restriction fragment length polymorphism (ARFLP) essentially as previously described[37]. Briefly, for each individual a polymerase chain reaction (PCR) was performed using 2 pmol each of the iNOS-F (5'-TGTAAACCAACTTCCGTGGTG-3') and iNOS-R (5'-174 GTCTCTGCGGGTCTGAAG-3') primers, 200 mmol dNTPs, 10 mmol Tris-HCl, pH 8.3, 50 mmol KCl, 1.5 mmol $MgCl_2$, 0.25 U of Hot start Taq DNA polymerase (Fermentas, Italy) and 50 ng of genomic DNA template in a final reaction volume of 25 μL. Each reaction was denatured at 95 ℃ for 5 min followed by 35 cycles of denaturation at 94 ℃ for 60 s, annealing at 58.5 ℃ for 60 s, and extension at 72 ℃ for 40 s. A final extension at 72 ℃ for 5 min was added to ensure complete amplification. Restriction digests were performed by incubating 10 μL of PCR product with 2 U of TSP 509I enzyme (New England Biolabs, Ipswich, MA, United States) in a final volume of 15 μL for 15 h at 65 ℃. Digestion products were separated on a 1.5% SDS-polyacrylamide gel, and visualized after staining with ethidium bromide. Results of the ARFLP analysis allowed us to differentiate between the 3 possible genotypes: (1) digestion of PCR amplicons from homozygous (CC) individuals generated 2 DNA fragments of 113 bp and 175 bp; (2) digestion of amplicons from heterozygous individuals (CT) yielded 3 DNA fragments of 113 bp, 142 bp, and 175 bp; and (3) digestion of amplicons from homozygous (TT) individuals yielded 2 DNA fragments of 113 bp and 142 bp.

H. pylori detection

Infection with *H. pylori* was detected by culturing gastric tissue collected during endoscopy. Identification of clinically isolated *H. pylori* strains was confirmed by Gram stain and urease activity. Additionally, all patients were also tested for infection using an *H. pylori* specific IgG enzyme linked immunosorbent assay (ELISA) (Diagnostic Automation, CA, United States) and by urease breath test (Bahar, Afshan, Iran). Given the difficulties in culturing *H. pylori*, especially from cancerous tissue, individuals were classified as infected with *H. pylori* if they gave a positive result by ELISA.

Table 1 Patient demographics and risk behavior

	Gastric cancer n = 159 (%)	Control n = 170 (%)	P-value
Age (yr)	61.32 ± 12.5	58.86 ± 14.2	0.098
Sex (male/female)	84/75	98/72	0.367
Marital status			
Single	7 (4.4)	3 (1.8)	
Married	149 (93.7)	166 (97.6)	0.002
Divorced	3 (1.9)	1 (0.6)	
Occupation			
Unemployed	4 (2.5)	1 (0.6)	
Employee	24 (15.1)	35 (20.5)	0.001
Housewife	46 (28.9)	74 (43.5)	
Others	85 (53.5)	60 (5.4)	
Smoker	33 (20.8)	22 (12.9)	0.015
Hot beverage	76 (47.8)	38 (22.4)	< 0.0001
Salty fish	15 (9.4)	14 (8.2)	0.09
Fast food	29 (18.2)	39 (22.9)	0.037
Pickles	88 (55.3)	97 (57.1)	0.62
H. pylori positivity	94 (59.1)	77 (45.3)	0.015
Positive family history	34 (21.4)	16 (9.4)	0.0034

H. pylori: *Helicobacter pylori*.

Statistical analysis

Fisher's exact test was used to compare the different *iNOS* allele types, and the frequency of each *iNOS* genotype in the patient population(s) was assessed using a χ^2 test. The difference in age between the 2 study groups was evaluated using a two-tailed Student's *t*-test. Deviation from the Hardy-Weinberg equilibrium was evaluated using a χ^2 test. To evaluate the risk factor(s) in the study populations, unconditional logistic regression models were applied to odds ratios (ORs) and 95% confidence intervals (CIs). For all statistical tests, a P value of less than 0.05 was considered significant.

RESULTS

Demographics and epidemiology of the patient population

Complete epidemiologic and demographic information for the study population is given in Table 1. Across the 329 study participants, ages ranged from 28 years to 86 years. Within the control group the mean patient age was 58.9 ± 14.2 years, whereas within the cancer group the mean patient age was 62.1 ± 12.6 years. There was no significant difference between the ages of patients in the two groups (P = 0.098). The control group was comprised of 98 male and 72 female patients while the cancer group was comprised of 84 male and 75 female patients. There was no significant difference between the distribution of male and female study participants in either group (P = 0.367). Patients were categorized by marital status as single, married, or divorced. The cancer group consisted of 7 single, 149 married, and 3 divorced individuals. In contrast, the control group consisted of 3 single, 166 married, and a single divorced patient. The breakdown of marital status between the case and control groups was significant (P = 0.002). The study participants were also broken down into different groups based on occupation. Patients were considered either unemployed, employed, or worked as a housewife; those participants whose employment status did not fall into one of these 3 categories were grouped into a single category, which was named "other". In the GC group, 4 of the study members were unemployed, 24 were employed, 46 were housewives, and the remaining 85 were in the other classification. Within the control group, 1 participant was unemployed, 35 were employed, 74 were housewives, and 60 were included in the other category. The distribution of patient occupation among the case group and the control group was significantly different (P = 0.001).

Risk factors associated with GC

In addition to gathering demographic information about the study participants, we also analyzed behaviors that are known to be associated with increased GC risk. Of the 159 patients who were diagnosed with GC, 33 were smokers whereas only 22 of the healthy controls reported smoking. Since smoking is an established risk factor for development of GC, perhaps it is not surprising that the increased number of smokers in the cancer group was statistically significant (P = 0.015). Another established risk factor for GC is consumption of hot beverages; drinking hot tea is specifically associated with increased risk of esophageal cancer in Iran[43]. Seventy-six participants in the cancer group reported drinking hot beverages on a regular basis. This number was significantly different than the 38 control patients who regularly drank hot beverages (P < 0.0001). Although consuming high amounts of dietary salt has been linked to GC[23-25], we found no significant difference in the number of patients who regularly ate salted fish among the two groups. Similarly, we found no significant difference in the number of patients who consumed pickles among the groups. However, more of the healthy controls reported regularly eating fast food than did the participants who developed GC. In contrast to the other 2 food criteria, this difference was significant (P = 0.037).

Among the most widely recognized factors that are associated with GC risk are *H. pylori* infection and a family history of cancer. Among the control group, only 18 patients had a family history of GC. In contrast, 34 participants in the cancer group reported having a family history of GC (P = 0.003). As it is the most commonly associated risk factor for GC, we next compared the number of *H. pylori*-positive individuals in both of our study groups. In the cancer group, nearly 60% of the study participants tested positive for *H. pylori*. Compared to this relatively high infection rate, the percentage of healthy controls who tested *H. pylori*-positive (45%) was significantly less (P = 0.015).

Frequency and distribution of C150T inducible nitric oxide synthetase polymorphisms

Having established the relative risk factors of our population, we next sought to determine the distribution of the

Table 2 Allele and genotype frequency distribution of C150T polymorphism of the inducible nitric oxide synthetase gene in patients with gastric cancer and controls *n* (%)

iNOS C150T	Gastric cancer *n* = 159	Controls *n* = 170	Odds ratio	95%CI	*P*-value
CC	89 (56)	92 (54.1)	1.00[1]		
CT	59 (37.1)	72 (42.4)	0.86	0.54-1.35	0.5
TT	11 (6.9)	6 (3.5)	1.917	0.68-5.41	0.21
C allele	237 (74.5)	256 (75.3)	1.00[1]		
T	81 (25.5)	84 (24.7)	1.05	0.74-1.49	0.78
TT + CT	70 (44)	78 (45.9)	0.94	0.61-1.45	0.77
HWE	0.796	0.07			

[1]The first allele or genotype is the reference for statistical analysis. iNOS: Inducible nitric oxide synthetase; CC: Homozygous CC genotype; CT: Heterozygous CT genotype; TT: Homozygous TT genotype; HWE: Hardy Weinberg Equilibrium; CI: Confidence interval.

possible C150T *iNOS* genotypes among the two study groups (Table 2). Patients were genotyped essentially as described[37]; as reported in Japanese populations[36], the distribution of these specific *iNOS* polymorphisms in the control and cancer patient groups fell within the Hardy-Weinberg Equilibrium ($P = 0.07$ and $P = 0.796$, respectively). Among the 159 members of the GC group, 89 were homozygous CC, 59 were heterozygous CT and 11 were homozygous TT. The distribution of allele types within this group was not significantly different when compared to the study control group, which contained 92 homozygous CC, 72 heterozygous CT, and 6 homozygous TT individuals. There was also no difference in the frequency of each individual allele (C or T) found within the two study populations or the frequency of mutant (either CT or TT) genotypes between the GC and control groups.

CT and TT inducible nitric oxide synthetase polymorphisms contribute to GC risk in H. pylori-infected individuals

Since there was no significant difference in the frequency or distribution of *iNOS* C150T alleles in our two patient populations, we next examined any potential epidemiological interaction between *H. pylori* infection and the particular *iNOS* C150T genotype within the 159 GC patients. As shown in Table 3, the number of GC patients who were infected with *H. pylori* ($n = 94$) was slightly higher than the number of patients who were *H. pylori*-negative ($n = 65$). Among the 94 individuals who were infected with *H. pylori*, 46.8% were homozygous CC, 42.6% were heterozygous CT, and the remaining 10.6% were homozygous TT. In contrast, the *H. pylori*-negative group consisted mostly of the homozygous CC genotype (67.7%), followed by the heterozygous CT genotype (29.2%) and the TT genotype (3.1%). When compared to the non-*H. pylori* infected group, GC patients who had a heterozygous CT genotype and were also infected with *H. pylori* were 2.1 times more at risk of developing GC (OR = 2.1, $P = 0.03$, χ^2 test). Similarly, having a homozygous TT genotype and being infected with *H. pylori* also led to an increased risk of developing GC; the risk associated with having the homozygous TT genotype was more than twice the risk associated with having only a single T allele (OR = 5.0). This association was also significant ($P = 0.029$, χ^2 test). Similarly, the presence of either mutant allele (CT or TT) increased the risk of GC nearly 2.5-fold (OR = 2.4, $P = 0.01$, χ^2 test). Taken together, these results indicate that for individuals who are infected with *H. pylori*, carrying either a CT or TT *iNOS* polymorphism at position 150 significantly increases the risk of developing GC. Importantly, unlike those patients within the GC group, there was no significant association between *iNOS* genotype, *H. pylori* infection and risk of developing GC in the control group (Table 3).

Table 3 Allele and genotype frequency distribution of C150T polymorphism of the inducible nitric oxide synthetase gene in *Helicobacter pylori*[3] *n* (%)

	H. pylori+	*H. pylori*-	OR[2]	95%CI	*P*-value
iNOS C150T	*n* = 94	*n* = 65			
GC patients					
CC	44 (46.8)	44 (67.7)	1.00[1]		
CT	40 (42.6)	19 (29.2)	2.1	1.06-4.19	0.03
TT	10 (10.6)	2 (3.1)	5	1.03-24.14	0.029
C	128 (68.1)	107 (82.3)	1.00[1]		
T	60 (31.9)	23 (17.7)	2.2	1.26-3.76	0.004
TT + CT	50 (53.2)	21 (32.3)	2.4	1.23-4.6	0.01
Controls	*n* = 77	*n* = 93			
CC	40 (51.9)	52 (55.9)	1.00[1]		
CT	36 (46.8)	36 (38.7)	1.3	0.7-2.41	0.41
TT	1 (1.3)	5 (5.4)	0.26	0.03-2.31	0.197
C	116 (75.3)	140 (75.3)	1.00[1]		
T	38 (24.7)	46 (24.7)	0.99	0.61-1.64	0.99
TT + CT	37 (48.05)	41 (44.1)	1.2	0.64-2.15	0.6

[1]The first allele or genotype is the reference for statistical analysis; [2]Adjusted for age and gender; [3]Infected or non-infected patients with and without GC. GC: Gastric cancer; iNOS: Inducible nitric oxide synthetase; CC: Homozygous CC genotype; CT: Heterozygous CT genotype; TT: Homozygous TT genotype; C: Number of individual C alleles in the group; T: Number of individual T alleles in the group; HWE: Hardy Weinberg Equilibrium; OR: Odds ratio; CI: Confidence interval; *H. pylori*: *Helicobacter pylori*.

DISCUSSION

The study of *H. pylori*-induced disease is complicated by the fact that the etiology of disease appears to result from the intricate interplay between bacterial virulence factors, host genetics and environmental components. Moreover, an increasing body of literature indicates that colonization by *H. pylori* may even be beneficial to some hosts[10-12]. Because of these facts, there is a pressing need to develop new diagnostics that will clearly identify those individuals most at risk for development of the most severe form of *H. pylori*-induced disease-GC. To this end, numerous studies have looked at the role of virulence factors, environmental risk factors and host genetic polymorphisms in disease development. While these studies have shed an enormous amount of light on development of GC, it is becoming increasingly more evident that vari-

ous factors play different roles based on the population being studied[13-21,23-25,27-30]. Thus, there is a need for larger population-based studies to define the role of particular factors in the disease process. Despite the high prevalence of GC and *H. pylori* infection in Northern Iran, there have been relatively few studies that have examined specific risk factors associated with disease development in this population. Of those that have been conducted, one of the studies that investigated the high rate of cancer development in this region concluded that the classic *H. pylori* virulence factors were not a good predictor of disease outcome[45]. Furthermore, the authors went on to suggest that "...the association between strain virulence markers and disease in Iraq but not Iran suggests that other host and environmental factors may be more important in the disease-prone Iranian population"[45]. Given this suggestion and the deficit in our knowledge, we set out to examine the contribution of particular behavioral factors and genetic polymorphism in the *iNOS* gene to GC development in the Iranian population. Using a case-controlled study design we identified a significant association of several factors with an increased risk for GC. Among the behavioral factors that were important, smoking and consumption of hot beverages were both associated with development of GC. Both of these factors have been previously identified as risk factors for cancer in other populations[36,37]. However, our study is the first to link consumption of hot beverages with GC development in this particular region.

In addition to behavioral factors and similar to other previously reported risk factors[46], we found that a familial history of GC was significantly associated with increased risk of GC development in our study population (Table 1). Also similar to at least one other study in this region[2], we found that infection with the gastric pathogen *H. pylori* significantly increased the risk of developing GC. Finally, we found that carrying either a heterozygous (CT) allele or a homozygous (TT) allele at position 150 of exon 16 of the *iNOS* gene combined with *H. pylori* infection resulted in a significant increase in GC development.

In humans, NO is used as an important signaling molecule. However, in the presence of increased and/or sustained levels of this molecule, the beneficial effects of NO can be overcome by deleterious processes such as DNA damage and chronic inflammation. These pathologic events may be catalyzed by increased iNOS activity, which leads to increased NO production and concomitant increases in the amount of downstream free radicals. As the major source of NO in humans, perhaps it is not surprising that even minor amino acid changes in the iNOS enzyme can affect the levels of NO. The (C150T) polymorphism found in exon 16 of the *iNOS* gene was previously suggested to affect activity of this enzyme, leading to increased production of NO[36]. Interestingly, in our or other populations[36,37], the *iNOS* polymorphism alone was not a risk factor for cancer development. This fact suggests that while the C150T polymorphism may affect NO production, it does not do so to such an extent as to induce cancer development in isolation. Instead, a co-carcinogenic factor is required to synergistically interact in such a way as to promote disease progression. Within the population of Northern Iran, this co-carcinogenic factor appears to be *H. pylori* infection (Table 3). A similar result was also found within the Japanese population[36]. Alternatively, the *iNOS* polymorphism was suggested to act as a co-carcinogenic factor with past cigarette smoking and alcohol consumption within the Chinese population[37]. Thus, in multiple populations, iNOS polymorphism synergistically interacts with various factors to affect GC development.

While the molecular mechanism of synergistic interaction between the *iNOS* polymorphism and *H. pylori* infection is not clear, one plausible hypothesis is that since *H. pylori* infection alone is sufficient to increase NO levels in gastric tissue[47,48], the addition of a polymorphism that also increases iNOS enzyme activity could increase the amount of NO in the local tissue to a further degree. In turn, this combined increase in NO could skew the gastric mucosa toward a pro-inflammatory response and increase the likelihood of carcinogenic cellular damage. It is interesting to note that *H. pylori* infection alone creates a chronic pro-inflammatory environment within the gastric mucosa. Furthermore, it has been well documented that the ability of *H. pylori* to establish a persistent infection depends on maintenance of the proper balance between the host immune system and the bacterium (reviewed in[49]). In patients with either the CT or TT *iNOS* polymorphisms, the increased levels of NO may upset this delicate balance, thus perpetuating pathological and carcinogenic events.

H. pylori-mediated cancer development is a complex and multi-faceted process that involves a combination of bacterial, host, and environmental factors. Moreover, infection with *H. pylori* is inversely correlated with development of esophageal cancer[12], and is thought to provide protection against development of autoimmune disorders such as asthma[11]. Thus, the apparent benefit(s) of asymptomatically harboring this organism brings to bear the question of whether it is best to treat all patients for *H. pylori* infection; if not, the question becomes-how do we identify individuals most at risk for developing serious disease? One possible way to differentiate patients who have a high risk for disease development from those who are low-risk is to specifically examine bacterial, host-genetic and environmental factors that have been shown to be associated with cancer development in *H. pylori*-infected individuals. By screening individual patients for these three types of markers, it may be possible to specifically identify and treat those individuals who are at most risk for developing severe disease while reducing the risk of losing the apparent protective effects of *H. pylori* infection in low-risk individuals.

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COMMENTS

Background

As gastric cancer (GC) remains a serious source of morbidity and mortality worldwide, the identification of individuals most at risk for disease development is a major concern.

Research frontiers

Recent studies have highlighted the importance of polymorphisms in host genes as a risk factor for GC development. However, as many of these studies have been performed on limited patient populations, it remains unclear whether these polymorphisms are important across diverse ethnic groups and geographic areas. In this study, the authors identified an increase in GC risk in *Helicobacter pylori* (*H. pylori*)-infected Iranian patients who harbored a CT or TT polymorphism at position 150 in the inducible nitric oxide synthetase (*iNOS*) gene.

Innovations and breakthroughs

To the best of our knowledge, this study describes the first known link between a host-genetic factor and GC risk in an Iranian population. In addition, this study is the first to define the importance of the *iNOS* C150T polymorphism in a non-Asian population.

Applications

The results of this study suggest that the C150T *iNOS* polymorphism is a potential marker for susceptibility to *H. pylori*-mediated gastric disease.

Peer review

This is a study aimed at investigating the association of the *iNOS* C150T polymorphism with *H. pylori* infection and GC risk among patients from a region of Iran. The authors present an interesting study from a region that has not been addressed very often in regard to *H. pylori* and its related pathologies.

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BRIEF ARTICLE

Endothelial precursor cells promote angiogenesis in hepatocellular carcinoma

Xi-Tai Sun, Xian-Wen Yuan, Hai-Tao Zhu, Zheng-Ming Deng, De-Cai Yu, Xiang Zhou, Yi-Tao Ding

Xi-Tai Sun, Xian-Wen Yuan, Hai-Tao Zhu, Zheng-Ming Deng, De-Cai Yu, Yi-Tao Ding, Department of Hepatobiliary Surgery, the Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing 210008, Jiangsu Province, China
Xi-Tai Sun, Xian-Wen Yuan, Hai-Tao Zhu, Zheng-Ming Deng, De-Cai Yu, Yi-Tao Ding, Institute of Hepatobiliary Surgery, Nanjing University, Nanjing 210008, Jiangsu Province, China
Hai-Tao Zhu, Zheng-Ming Deng, School of Medicine, Nanjing University, Nanjing 210008, Jiangsu Province, China
Xiang Zhou, Department of Pathology, the Affiliated Drum Tower Hospital, School of Medicine, Nanjing University, Nanjing 210008, Jiangsu Province, China
Author contributions: Sun XT and Ding YT designed the research; Yuan XW, Zhu HT, Deng ZM, Yu DC and Zhou X performed the research; Sun XT analyzed the data and wrote the paper.
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Correspondence to: Yi-Tao Ding, Professor, Department of Hepatobiliary Surgery, the Affiliated Drum Tower Hospital of Nanjing University Medical School, No. 321 Zhongshan Road, Nanjing 210008, Jiangsu Province,
China. yitaoding@hotmail.com
Telephone: +86-25-83304616 Fax: +86-25-83317016
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Abstract

AIM: To investigate the role of bone marrow-derived endothelial progenitor cells (EPCs) in the angiogenesis of hepatocellular carcinoma (HCC).

METHODS: The bone marrow of HCC mice was reconstructed by transplanting green fluorescent protein (GFP) + bone marrow cells. The concentration of circulating EPCs was determined by colony-forming assays and fluorescence-activated cell sorting. Serum and tissue levels of vascular endothelial growth factor (VEGF) and colony-stimulating factor (CSF) were quantified by enzyme-linked immunosorbent assay. The distribution of EPCs in tumor and tumor-free tissues was detected by immunohistochemistry and real-time polymerase chain reaction. The incorporation of EPCs into hepatic vessels was examined by immunofluorescence and immunohistochemistry. The proportion of EPCs in vessels was then calculated.

RESULTS: The HCC model was successful established. The flow cytometry analysis showed the mean percentage of CD133CD34 and CD133VEGFR2 double positive cells in HCC mice was 0.45% ± 0.16% and 0.20% ± 0.09% respectively. These values are much higher than in the sham-operation group (0.11% ± 0.13%, 0.05% ± 0.11%, n = 9) at 14 d after modeling. At 21 d, the mean percentage of circulating CD133CD34 and CD133VEGFR2 cells is 0.23% ± 0.19%, 0.25% ± 0.15% in HCC model *vs* 0.05% ± 0.04%, 0.12% ± 0.11% in control. Compared to the transient increase observed in controls, the higher level of circulating EPCs were induced by HCC. In addition, the level of serum VEGF and CSF increased gradually in HCC, reaching its peak 14 d after modeling, then slowly decreased. Consecutive sections stained for the CD133 and CD34 antigens showed that the CD133+ and CD34+ VEGFR2 cells were mostly recruited to HCC tissue and concentrated in tumor microvessels. Under fluorescence microscopy, the bone-marrow (BM)-derived cells labeled with GFP were concentrated in the same area. The relative levels of *CD133* and *CD34* gene expression were elevated in tumors, around 5.0 and 3.8 times that of the tumor free area. In frozen liver sections from HCC mice, cells co-expressing CD133 and VEGFR2 were identified by immunohistochemical staining using anti-CD133 and VEGFR2 antibodies. In tumor tissue, the double-positive cells were incorporated into vessel walls. In immunofluorescent staining. These CD31 and GFP double positive cells are direct evidence that tumor vascular endothelial cells (VECs) come partly from BM-derived EPCs. The proportion of GFP CD31 double positive VECs (out of all VECs) on day 21 was around 35.3% ± 21.2%. This is much higher than the value recorded on day 7 group (17.1% ± 8.9%). The expression of

intercellular adhesion molecule 1, vascular adhesion molecule 1, and VEGF was higher in tumor areas than in tumor-free tissues.

CONCLUSION: Mobilized EPCs were found to participate in tumor vasculogenesis of HCC. Inhibiting EPC mobilization or recruitment to tumor tissue may be an efficient strategy for treating HCC.

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Key words: Hepatocellular carcinoma; Angiogenesis; Endothelial progenitor cells; Bone-marrow cells; Orthotropic hepatic cancer model

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most vascular solid tumors, and neovascularization plays an important role in HCC development. In HCC, neovascularization status is correlated with disease progression and patient prognosis. Endothelial progenitor cells (EPCs) have been proved to be the main source of adult neovascularization. Until now, there has not been any data regarding the role of EPCs in HCC neovascularization. This study is the first to prove that EPCs incorporate into vessels directly and play an important role in HCC neovascularization.

It has been confirmed that neovascularization is vital to further multiplication, metastasis, and recurrence in malignant tumors. Without exogenous blood vessels, tumors stop growing and enter a state of suspended animation at about 2-3 cubic millimeters. The acquisition of angiogenic capacity is considered a sign of tumor development and of metastasis[1].

Recently, increasing amounts of data have revealed that bone-marrow (BM)-derived EPCs can foster the initiation and maintenance of angiogenic processes by integrating into developing vasculature under hypoxic conditions and into tumors. In 1997, EPCs were first isolated using anti-CD34 monoclonal antibodies. CD34-antigen, however, could not distinguish immature EPCs from mature circulating endothelial cells (CECs) because CD34 is expressed on both. With the discovery of CD133, an antigen restricted to immature EPCs, distinguishing between EPC and CECs has become easier[2]. VEFGR2 is another marker of EPCs. VEFGR2 signaling causes EPCs to differentiate. EPCs resemble embryonic angioblasts, which characteristically migrate, proliferate, and differentiate into mature endothelial cells[3]. In general, EPCs can be identified as cells that simultaneously express the cell surface markers CD133, CD34, and VEGFR2[4,5]. Santarelli *et al*[6] used mouse tumor models to demonstrate that BM-derived EPCs are greatly mobilized into the bloodstream, and home specifically to tumor tissues. In the tumor microenvironment, the recruited EPCs merged with the walls of a growing blood vessel, where they differentiated into endothelial cells and so promoted tumor growth. It has been reported that, during cancer proliferation, the number of circulating BM-derived progenitor cells increases dramatically. Circulating BM-derived EPCs have been used to determine the optimal dose of anti-angiogenic drugs, most successfully in breast cancer[7]. In addition, Taylor proved that high levels of circulating BM-derived progenitor cells are correlated with tumor metastatic status. Recently, the contribution of EPCs to tumor angiogenesis has also been reported in clinical tumor samples from 6 patients who received bone marrow transplants from donors of the opposite sex. The endothelial cells within the tumor vessels were of donor origin, as shown by the mismatched sex chromosomes[8].

At present, the significance of circulating EPCs and the contribution of EPCs to tumor vessels have been confirmed in only a few solid malignancies, such as malignant glioma[6], lung cancer[9], and mammal tumors[10]. However, the extent to which EPCs contribute to the generation of the tumor vessels varies significantly across different studies, from substantial to zero[11,12]. The role of EPCs in neovascularization has been the subject of great debate.

Tumor microvessel density is closely related to tumor growth, invasion, and metastasis and to patient prognosis[13,14]. It has been confirmed that the formation of new capillaries is essential to tumor development, and the status of angiogenesis is an important prognostic indicator in hepatocellular carcinoma[15,16]. There are only three papers in PUBMED, including our previous study[17], that compare the circulating EPC levels of HCC patients to those of healthy controls or those of liver cirrhosis patients[18,19]. We established an orthotropic hepatic cancer model, observed the dynamic changes of circulating EPCs, and determined whether BM-derived EPCs contributed to tumor vessels directly. We also calculated the incorporation rate of the EPCs in vessels. All this information is extremely important to evaluating the role of EPCs in neovascularization in HCC.

MATERIALS AND METHODS

Orthotropic hepatic cancer mouse model

For BM reconstruction, C57BL/6 male mice aged 8-10 wk were lethally irradiated (10 Gy) and transplanted with BM cells collected from green fluorescent protein (GFP)-C57BL/6 transgenic mice[6]. Four weeks later, the HCC

model showed a GFP positive rate of circulating nucleated cells of over 90%, suggesting full BM recovery. The mice were then used to construct an orthotropic hepatic cancer mice model by intra-hepatic injection of 1×10^5-2×10^5 H22 hepatoma cells (given to us by Dr. Ru-Tian Li of the Nanjing University Institute of Oncology, China). Sterile extract H22 hepatoma cells, normal saline to adjust the number of cancer cells to 106/mL for use. Intraperitoneal injection of sodium pentobarbital anesthesia C57BL/6 mice, disinfection shop towel, in the middle upper abdomen about the xiphoid mainly as a 1 cm incision into the abdominal cavity. Gently press out part of the lobe with bilateral costal arch, wet gauze pad in the bottom lobe to protect, and then with 1 mL medical syringe needles inserted along the long axis of lobe about 0.5 cm, the slow injection of H22 hepatoma cells 0.1 mL (cell number 1×10^5-2×10^5). Pull out the needle at the same time, rapidly at the pressure in the eye of a needle, and gently press on it with a cotton for 1 min to stop bleeding. Carefully place the liver back into the abdominal cavity. The control group received same volume of phosphate buffered solution (PBS). All mice were from the national genetically engineered mouse resource bank of China.

Degree of hepatocellular carcinoma mobilization

Flow cytometry (FCM) was used to evaluate the degree of EPC mobilization. Three hundred microliters of peripheral blood was obtained from the retroorbital plexus at 3 d, 7 d, 14 d, and 21 d after the establishment of the orthotropic HCC model. Erythrocytes were split by red blood cell lysis buffer. Mononuclear cells were incubated for 30 min at 4 ℃ with Alexa Fluor488 conjugated anti-CD133, Alexa Fluor647 conjugated anti-CD34, PE-conjugated anti-VEGFR2 (eBioscience, United States). Appropriate fluorochrome-conjugated isotypes were used as controls. In addition, serum concentration of vascular endothelial growth factor (VEGF), platelet-derived growth factor [colony-stimulating factor (CSF)] was tested by enzyme-linked immunosorbent assay kit (ADL, United States) following the collection of serum at the same times.

Identification of BM-derived CD133+CD34+VEGR2+ cells in tissue

Liver tissue was obtained from the GFP labeled BM-HCC model on day 14. After paraformaldehyde perfusion and gradient dehydration, consecutive frozen liver tissue sections 2 μm in thickness were cut in a cryostat, fixed, blocked, and incubated at 4 ℃ overnight with primary antibody rabbit anti-mouse CD133 (1:200), VEGFR2 (1:100, Abcam, United States), rat anti-mouse CD34 (1:250, ebioscience, United States) respectively. Secondary antibody was horseradish peroxidase. Positive reactions were visualized with diaminobenzidine solution followed by counterstaining with hematoxylin. Negative controls were obtained by substituting the primary antibodies with PBS.

Contribution of BM derived EPCs to vessels

Consecutive 2 μm frozen liver tissue sections were cut as stated above. Sections were blocked and then incubated overnight with rat anti-mouse CD31(1:800, eBiosicence, United States). After washing in PBS, slices were incubated with secondary antibodies Alexa Fluor 488-conjugated rabbit anti-rat IgG antibody (1:1000, molecular probes). 4',6'-diamidino-2-phenylindole hydrochloride was used to dry the nucleus. Normal mice given PBS-substituted for antibodies as a negative control. Double-labeled immunofluorescence staining sections were evaluated using fluorescence microscopy.

Proportion of EPCs in vessels

Mouse livers were obtained at 7 d or 21 d after the establishment of the GFP-BM HCC model. Two-microliter frozen anti-CD31 immunofluorescence staining sections were created as stated above. Specimens were analyzed microscopically by two independent observers who counted the number of BM-derived vascular ECs (VECs) co-expressing GFP, CD31, and total VECs in 15 random high-power fields. The quantitative contribution of EPCs to total VECs is expressed as percentages[20].

Expression of intercellular adhesion molecule 1, vascular adhesion molecule 1, and VEGF protein in tissue

Liver tissue was obtained from the GFP-labeled BM-HCC model on day 14. Conventionally processed and embedded sections cut at a thickness of 3 μm were deparaffinized, blocked, and incubated at 4 ℃ overnight with primary antibody: rabbit anti-mouse intercellular adhesion molecule 1 (ICAM1) (1:50, Protein Tech Group, United States), vascular adhesion molecule 1 (VCAM1) (1:200 Santa Cruz Biotech, Santa Cruz, CA, United States), VEGF (1:1000 Abcam, City, State, United States). The secondary antibody was horseradish peroxidase. Positive reactions were visualized with diaminobenzidine solution followed by counterstaining with hematoxylin. Negative controls were obtained by substituting the primary antibodies with PBS.

Levels of CD133 and CD34 genes in tissue

The levels of actin and CD133 mRNAs in the tumor and tumor-free areas were measured by real-time quantitative polymerase chain reaction (PCR). RNA was extracted from the livers with Trizol (Invitrogen, United States). cDNA was generated using Takara Primer ScriptTM 1st stand cDNA synthesis kits (JP). The sequences of primers were designed and synthesized by Takara. Primers for CD133 (5'-AACGTGGTCCAGCCGAATG-3', 5'-TCCCAGGATGGCGCAGATA-3'), CD34 (5'-ACCCACCGAGCCATATGCTTAC-3', 5'-GATACCCTGGGCCAACCTCA-3'), β-actin (5'-CAT CCG TAA AGA CCT CTA TGC CAA C-3', 5'-ATG GAG CCA CCG ATC CAC A-3'). The PCR reactions were performed in the light of instruction of SYBR Premix Ex TaqTM Kit (Takara, Japan) using the Stratagene

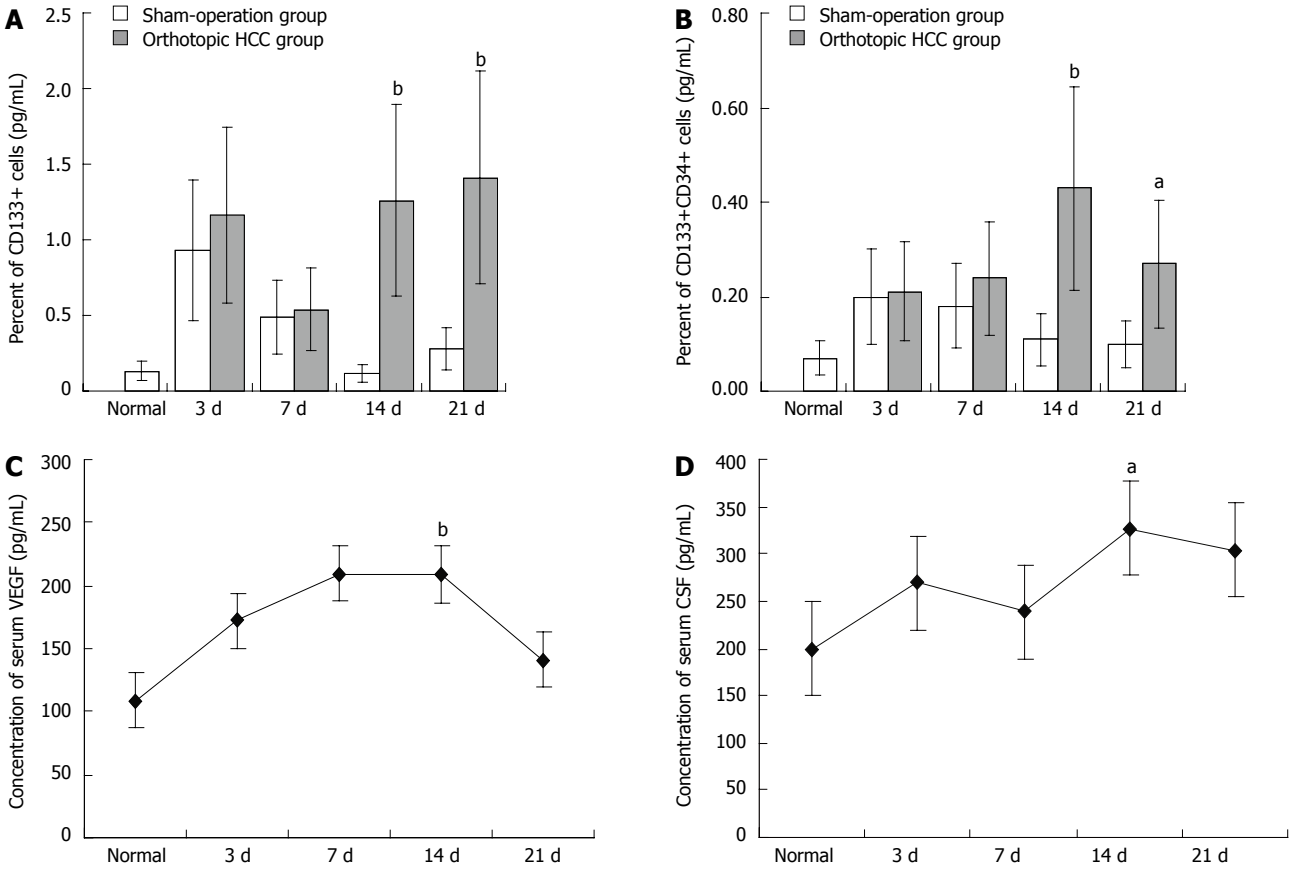


Figure 1 Mobilization of endothelial progenitor cells into circulation. A, B: Flow cytometry analysis of circulating endothelial progenitor cells in hepatocellular carcinoma (HCC) mice (day 14, $n = 7$; day 21, $n = 9$) *vs* sham-operation group (day 14, $n = 9$; day 21, $n = 11$). Normal = normal mice; C, D: Dynamic changes in serum levels of vascular endothelial growth factor and colony-stimulating factor in HCC mice ($n = 8$). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal control ($n = 8$). CSF: Colony-stimulating factor; VEGF: Vascular endothelial growth factor.

Mx3000P QPCR System (Bioscience Corporation). Each sample was measured in duplicate. The relative level of expression was determined by DDCt normalized to endogenous control (β-actin).

Statistical analysis

Statistical comparisons were performed using the *t*-test and analysis of variance when data were normally distributed or with separate variance estimation *t*-test or rank sum test. All statistical procedures were performed using SPSS 15.0. All data are presented as mean ± SD. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Establishment of BM orthotopic hepatocellular carcinoma model

The successful establishment of the HCC model was confirmed by a pathological exam confirming classic clinicopathological characteristics of HCC, intratumor hemorrhage, intrahepatic metastasis, and tumor neovascularization.

Mobilization of EPCs into circulation

The FCM analysis showed the mean percentage of circulating CD133CD34 and CD133VEGFR2 double positive cells in HCC mice to be 0.45% ± 0.16% and 0.20% ± 0.09% ($n = 7$), respectively. These values are much higher than in the sham-operation group (0.11% ± 0.13%, 0.05% ± 0.11%, $n = 9$) at 14 d after modeling. At 21 d, the mean percentage of circulating CD133CD34 and CD133VEGFR2 cells is 0.23% ± 0.19%, 0.25% ± 0.15% in HCC model *vs* 0.05% ± 0.04%, 0.12% ± 0.11% in control. The mobilization of BM cells may have been a response to the operation. Compared to the transient increase observed in controls, the higher level of circulating EPCs were induced by HCC (Figure 1A, B). In addition, the level of serum VEGF and CSF increased gradually in HCC, reaching its peak 14 d after modeling, then slowly decreased (Figure 1C, D).

Recruitment of EPCs to hepatocellular carcinoma tissue

Consecutive sections stained for the CD133 and CD34 antigens showed that the CD133+ and CD34+ VEGFR2 cells were mostly recruited to HCC tissue and concentrated in tumor microvessels (Figure 2A). Under fluorescence microscopy, the BM-derived cells labeled with GFP were concentrated in the same area (Figure 2B). The relative levels of CD133 and CD34 gene expression were elevated in tumors, around 5.0 and 3.8 times that of the tumor free area (Figure 2C, D). As determined by protein and gene expression levels, EPCs in HCC mice were recruited to tumors and tumor microvessels. The results of each three trials were largely consistent with each other.

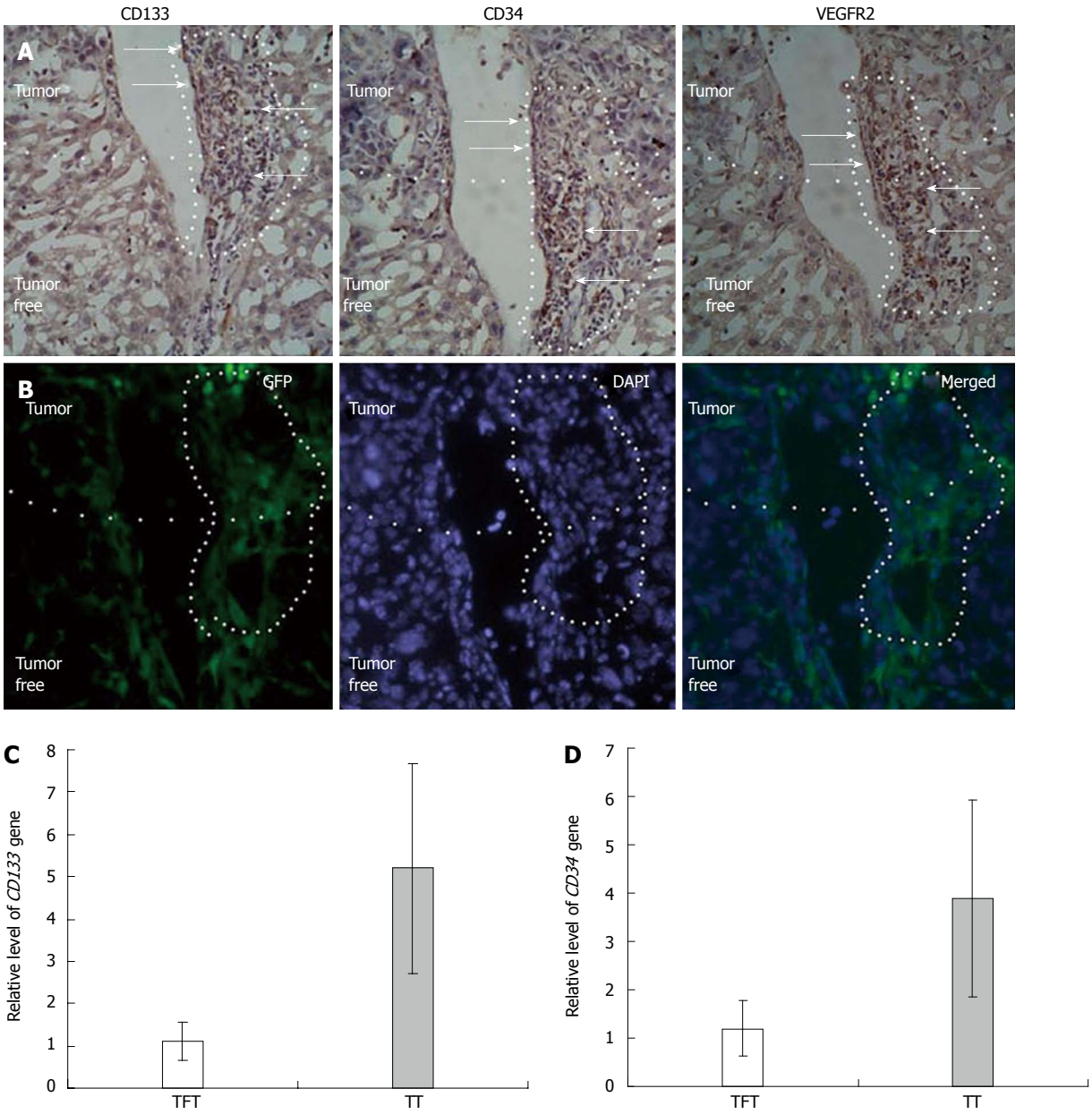


Figure 2 Recruitment of endothelial progenitor cells to hepatocellular carcinoma tissue. A: Distribution endothelial progenitor cells (EPCs) in tumor and tumor-free tissue in four consecutive 2 μm sections (arrows: Cells expressing the indicated antigens and recruited to tumors and tumor vessels); B: Bone-marrow cells labeled by green fluorescent protein recruited to tumors and tumor vessels, occupying the same position as antigen positive cells [blue regions: Nuclei stained by DAPI (frozen section, 2 μm, magnification 200)]; C: Relative levels of CD133 mRNA in tumor tissue (n = 7) and tumor-free tissue (n = 6); D: Relative level of CD34 mRNA in tumor tissue (n = 7) and tumor-free tissue (n = 7). TFT: Tumor-free tissues; TT: Tumor tissues; DAPI: 4',6'-diamidino-2-phenylindole hydrochloride; GFP: Green fluorescent protein.

Contribution of BM-derived EPCs to tumor blood vessels

In frozen liver sections from HCC mice, cells co-expressing CD133 and VEGFR2 were identified by immunohistochemical staining using anti-CD133 and VEGFR2 antibodies. In tumor tissue, the double-positive cells were incorporated into vessel walls (Figure 3C). In immunofluorescent staining, an antigen specific to mature endothelial cells CD31[21] was used to identify VECs. The image data show that cells co-expressing CD31 and GFP existed not only in sinusoid endothelial cells (Figure 3A) but also in hepatic veins (Figure 3B). These CD31 and GFP double positive cells are direct evidence that tumor VECs come partly from BM-derived EPCs. The proportion of GFP CD31 double positive VECs (out of all VECs) on day 21 was around 35.3% ± 21.2%. This is much higher than the value recorded on day 7 group (17.1% ± 8.9%) (Figure 3D).

Expression of ICAM1, VCAM1, and VEGF in tumor tissue

For further understanding the mechanism by which BM-derived EPCs home to tumors, we detected the expres-

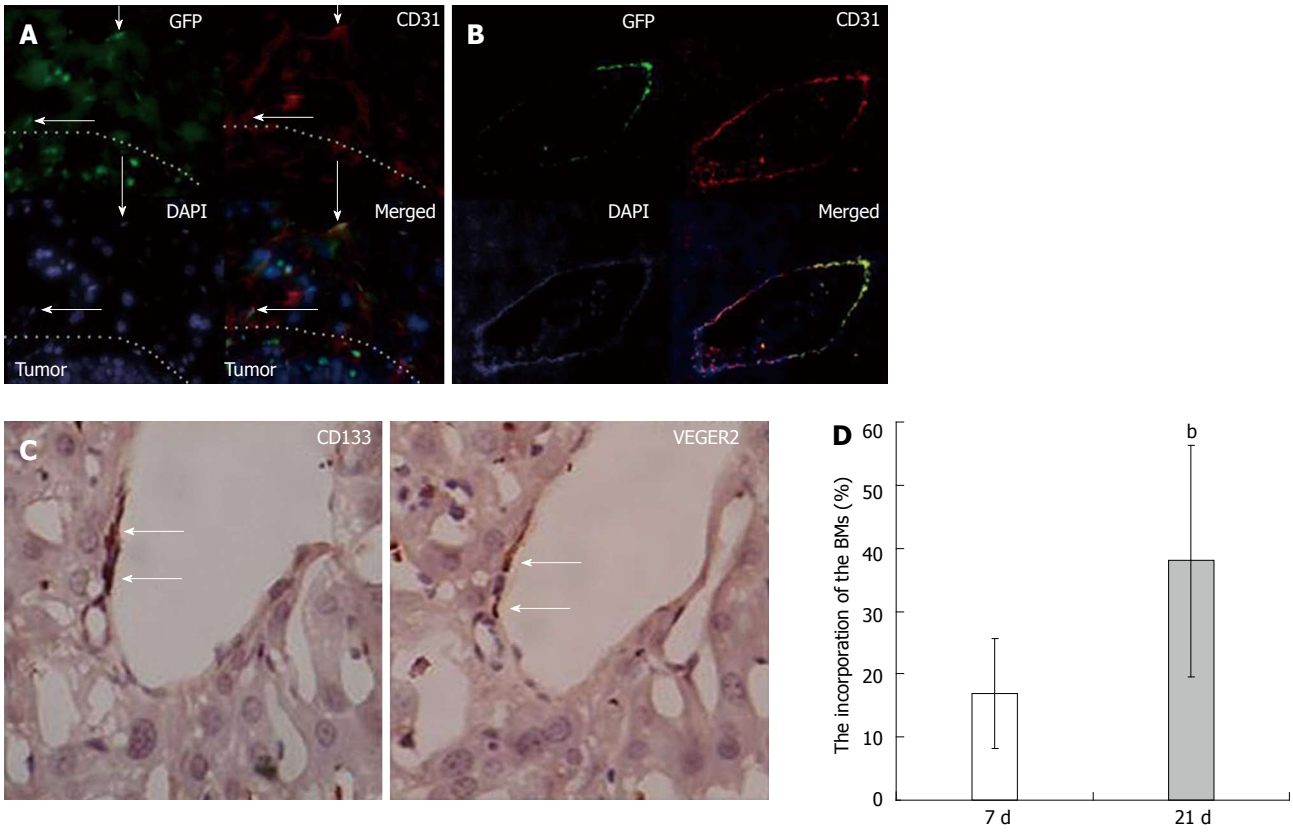


Figure 3 Bone-marrow-derived endothelial progenitor cells were found to contribute to vessel formation in hepatocellular carcinoma tissues. A: Bone marrow derived endothelial progenitor cells (EPCs) incorporated into peritumoral vessels; B: Distribution of CD133 and VEGFR2 antigens in hepatocellular carcinoma (HCC) liver vessels (Arrows: Double-positive cells, CD133+ VEGFR2+ cells incorporating into microvessels in the liver. Magnification, 400); C: Bone-marrow (BM)-derived EPCs incorporated into hepatic veins [Red: Mature vascular endothelial cells (VECs) are marked by CD31; Green: BM derived cells expressing green fluorescent protein (GFP); Blue: Nuclei stained by DAPI; Arrows: VECs co-expressing GFP and CD31, frozen, HCC, 2 μm, magnification 400]; D: The incorporation rate of the EPCs in HCC blood vessels at 7 d *vs* that observed at 21 d. $^{b}P < 0.01$ *vs* that observed at 21 d, $n = 15$. DAPI: 4',6'-diamidino-2-phenylindole hydrochloride.

sion of ICAM1, VCAM1, and VEGF in tumor tissues and tumor-free areas using immunohistochemistry. The results showed that the expression of ICAM1, VCAM1, and VEGF was higher in tumor areas than in tumor-free tissues (Figure 4).

DISCUSSION

Enhanced neovascularization is the most obvious clinicopathological characteristic of HCC, and tumor microvascular density is closely related to HCC progression and patient prognosis[2,22]. Although studies in some tumor models have shown that EPCs are mobilized into peripheral blood and contribute to tumor vascularization, to date, only Hong Kong University, our hepatic cancer institute, and the Medical University of Vienna have successively demonstrated that levels of circulating EPCs increase significantly and are correlated to clinical outcome in patients with HCC. The role of EPCs in HCC neovascularization is not currently clear. Based on our previous clinical findings[17,23], we established an orthotopic HCC model and used GFP as tracer to evaluate the role of EPCs in HCC-related angiogenesis[24,25]. According to PUBMED search, this is the first article to show direct visual evidence proving that EPCs promote angiogenesis by differentiating into vascular endothelia cells in HCC.

Thus far, the clinical significance of circulating EPCs has been confirmed in only a few solid malignancies, such as neurospongioma[25], hepatic cancer[17-19], breast cancer[26,27] and ovarian cancer[28]. Levels of circulating BM-derived stem cells have been used to determine the optimal dose of anti-angiogenic drugs, most successfully in breast cancer patients[7]. In the current investigation, which, unlike our previous clinical study, does not involve any interference from cirrhosis, it is much clearer that the mobilization of EPCs and the up-regulation of the BM-mobilization-related factors VEGF and CSF are the results of the development of HCC (Figure 1). Though wounds and injuries also can mobilize EPCs into the blood[29], the controls in this study showed only transient increases in, VEGF and CSF, and levels of circulating EPCs remained relatively low[30]. In terminal stage of the tumor, the decline in levels of serum cytokines and circulating EPCs may be the result of a disordered environment and systemic failure.

Characterization of EPCs, proof-of-principle strategies design to study the importance of vasculogenesis in tumors are current impediments. CD34-antigen could not distinguish immature EPCs from mature CECs. Recently, scientists discovered an antigen restricted to immature

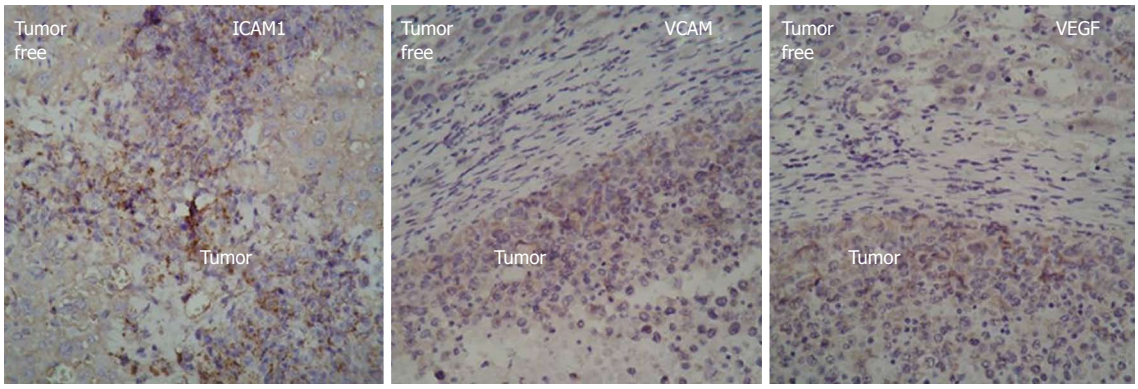


Figure 4 Expression of intercellular adhesion molecule 1, vascular adhesion molecule 1, and vascular endothelial growth factor in tumor tissues and tumor-free tissues as detected by immunohistochemistry (paraffin, liver, 2 μm, magnification, 200).

EPCs named CD133 which can distinguish between EPC and CECs. VEFGR2 is another marker of EPCs. VEFGR2 signaling causes EPCs to differentiate. EPCs resemble embryonic angioblasts, which characteristically migrate, proliferate, and differentiate into mature endothelial cells. Generally, EPCs can be identified as cells that simultaneously express the cell surface markers CD133, CD34, and VEGFR2.

In this study, we used GFP as a tracer because that tumor growth is accompanied by the formation of tumor blood vessels, we hypothesized that EPCs could home to liver tumors specifically. To test this hypothesis. The immunohistochemical image data of HCC tissue serial frozen sections showed many cells expressing EPC-specific antigens CD133, CD34, and VEGFR2, and these were concentrated in tumor tissues and tumor vessels (Figure 2A). Relative to tumor-free tissues, many BM-derived cells labeled by GFP specifically homed to tumor tissues and tumor vessels occupying the same positions as the CD133-, CD34-, and VEGFR2-positive cells (Figure 2B). This indicates that these antigen-positive cells come from bone marrow and co-express the specific antigens CD133, CD34, and VEGFR2. Real-time PCR analysis showed the levels of mRNA for stem cell markers CD133 and CD34 were around 5 and 3 times higher, respectively, than in tumor free tissues (Figure 2C). These results may be due to the fact that CD34 is expressed in both mature and immature cells, while CD133 is limited to immature cells (Figure 3B). EPCs' tropism for malignant tissues can also be observed in other cancer models, in which ICAM-1 is considered the main endothelial surface receptor responsible for recruitment of EPCs[31]. VEGF is believed to be the most important and essential mobilization factor in the induction of matrix metalloproteinase-9 (MMP-9) expression in bone marrow cells (BMCs). MMP-9, in turn, cleaves and activates the VEGF receptor of BMCs, permitting the transfer of BMCs and mobilizing EPCs into circulation[32]. In our HCC model, besides up-regulated VEGF, the levels of VEGF and ICAM1 in tumor tissue also increased significantly. This is consistent with our previously published clinical results[19]. From this, we can reasonably infer that, during tumor growth, HCC tissue releases mobilization factors, such as VEGF, into circulation. As the matrix degrades, BM-derived EPCs migrate into the liver through the bloodstream. Under the chemoattractive influence of locally high VEGF content VEGF and cellular adhesive molecules, the EPCs become come to rest in the hepatic tumor area.

Until now, the contribution of EPCs to blood vessel formation has been proved in only a few types of cancer, including malignant glioma[26] and Ewing's sarcoma[33,34]. We have identified and confirmed the clinical significance of mobilized EPCs in HCC[17]. Here, we directly prove that EPCs contribute to the formation of new HCC vessels not only in hepatic sinusoids (Figure 3A) but also in the veins of the liver (Figure 3C). Moreover, immunohistochemical image data has directly shown that cells co-expressing CD133 and VEGFR2 incorporate into live vessels (Figure 3D). This indicates that BM-derived EPCs stimulate tumor growth by promoting neovascularization. In addition, the incorporation rate of EPCs on day 21 was found to be much higher than on day 7. In this sense, the role of EPCs in neovascularization seem to become more important as HCC growth progresses (Figure 4C). Mature ECs have relatively limited proliferative capacity. In the current body of literature, the proportion of EPCs to VECs has been found to vary widely, from zero to more than 90%[11,12]. Other studies have concluded that EPCs do not contribute to the vascular endothelium and are not needed for tumor growth[35]. The fact that these studies used different tumor models may be the source of these different results.

Ever since 1971, anti-angiogenesis and anti-vascular treatments have emerged as a clinically promising anti-cancer strategy in HCC therapy. However, though anti-angiogenic therapies to HCC have been used clinically for a long time, the actual effect is not as satisfying as would be expected. Damaging tumor vasculature or blood embolization does lead to local metabolic disturbance, acidosis, hypoxia, and eventually to necrosis. This causes the release of soluble factor into the bloodstream by tumors directly and by the responding system indirectly. These soluble factors mobilize more EPCs into the blood and

promote the formation of new blood vessels to compensate for the lost blood supply. In fact, it has been found that tumor necrosis, hypoxia, and ischemia can all cause dramatic increases in serum levels of VEGF and in the number of EPCs in circulation EPCs[36-38]. These cells home to the vasculature of treated tumors and promote tumor neovascularization. In such cases, even if tumor vasculature is damaged and local angiogenesis is inhibited, BM-derived EPCs independently form the blood vessels necessary for tumor regrowth. The existence of compensatory cellular and molecular mechanisms inhibits the efficacy current targeted anti-angiogenic therapies. We suggest inhibiting the recruitment of circulating EPCs in concert with anti-vascular treatment. We have demonstrated previously that the number of circulating EPCs rises dramatically in patients with HCC. Now we further display that, during the course of HCC, large numbers of EPCs are mobilized and recruited to tumor areas, where they promote HCC angiogenesis by integrating directly into the tumor vessels directly. As HCC develops, the proportion of EPCs in vessels seems to rise gradually.

In view of current data, we have reason to believe that the BM is the important source of cells for neovascularization in HCC. We infer that blockage of EPC mobilization or recruitment to tumors may slow down hepatic cancer growth and improve the efficacy of therapies.

COMMENTS

Background

Neovascularization is the key process for tumor development, invasiveness and metastasis. It has been accepted that tumor-neovascularization raised in two ways, one is endothelial sprouting, another is bone marrow derived cells differentiating into mature vascular endothelial cells. Hepatocellular carcinoma (HCC) is one of the most vascular solid tumors, in which neovascularization plays an important role. The status of neovascularization in HCC correlates with the disease progression and patient's prognosis.

Research frontiers

At present, the significance of circulating endothelial progenitor cells (EPCs) and contribution of EPCs to tumor vessels have been confirmed only in a few solid malignancies, such as malignant glioma, lung caner and mammal tumor, however, the extent to which EPCs contribute to the generation of the tumor vessels is highly variable in different studies, from substantial to zero. So the role of EPCs on neovascularization has been argued a lot. Till now, there is no any data to evaluate the role of bone marrow cells (BMCs) in HCC neovascularization yet.

Innovations and breakthroughs

Ever since Professor Folkman put forward the dependence of tumor growth on angiogenesis in 1971, anti-angiogenesis or anti-vascular treatment has emerged as a clinically promising anti-cancer strategy in HCC therapies. However, though anti-angiogenic therapies to HCC such as chemotherapy and embolization have been in clinical application for a long time, the actual effect is not as satisfying as expected yet. It is well known HCC is a highly vascularized solid tumor. The authors have demonstrated that circulation BMCs/bone marrow-derived stem cells rise dramatically in patients with HCC previously in clinical cancer research, now the authors further display during the course of HCC, large number of BMCs was mobilized and recruited to tumor area, then promote HCC angiogenesis by integrating into tumor vessels directly.

Applications

The study results suggest that the BMCs play a prominent role in HCC neovascularization, and inhibiting EPC mobilization or recruitment to tumor tissue may be an efficient strategy for treating HCC.

Terminology

EPCs: Endothelial progenitor cells can differentiate into mature vascular endothelial cells for formation of new vessels (vasculogenesis).

Peer review

This is a good descriptive study to investigate the role of bone marrow-derived EPC in the angiogenesis of HCC. The results are interesting and suggest that mobilized EPCs were found to participate in tumor vasculogenesis of HCC and inhibiting EPC mobilization or recruitment to tumor tissue may be an efficient strategy for treating HCC.

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BRIEF ARTICLE

Coexistence of hyperlipidemia and acute cerebral ischemia/reperfusion induces severe liver damage in a rat model

Wei-Hong Gong, Wen-Xia Zheng, Jun Wang, Shi-Hui Chen, Bo Pang, Xia-Min Hu, Xiao-Lu Cao

Wei-Hong Gong, Wen-Xia Zheng, Jun Wang, Shi-Hui Chen, Bo Pang, Xia-Min Hu, Xiao-Lu Cao, Department of Pharmacology, College of Medicine, Wuhan University of Science and Technology, Wuhan 430065, Hubei Province, China

Author contributions: Gong WH, Zheng WX, Wang J and Chen SH contributed equally to this work; Gong WH, Zheng WX, Chen SH and Hu XM designed the research; Gong WH, Zheng WX, Chen SH, Pang B and Cao XL performed the research; Gong WH, Zheng WX, Wang J and Chen SH analyzed the data and wrote the paper.

Correspondence to: Xia-Min Hu, MD, PhD, Professor, Department of Pharmacology, College of Medicine, Wuhan University of Science and Technology, Wuhan 430065, Hubei Province, China. huxiamin@wust.edu.cn

Telephone: +86-27-68893640 Fax: +86-27-68893590

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Abstract

AIM: To investigate the correlation of hyperlipemia (HL) and acute cerebral ischemia/reperfusion (I/R) injury on liver damage and its mechanism.

METHODS: Rats were divided into 4 groups: control, HL, I/R and HL+I/R. After the induction of HL *via* a high-fat diet for 18 wk, middle cerebral artery occlusion was followed by 24 h of reperfusion to capture I/R. Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) were analyzed as part of liver function tests and liver damage was further assessed by histological examination. Hepatocyte apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The expression of genes related to apoptosis (caspase-3, bcl-2) was assayed by immunohistochemistry and Western blotting. Serum tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and liver mitochondrial superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and Ca^{2+} levels were measured to determine inflammatory and oxidative/antioxidative status respectively. Microsomal hydroxylase activity of the cytochrome P450 2E1 (CYP2E1)-containing enzyme was measured with aniline as the substrate, and CYP2E1 expression in the liver tissue and microsome was determined by immunohistochemistry and Western blotting respectively.

RESULTS: HL alone induced by high-fat diet for 18 wk resulted in liver damage, indicated by histopathological analysis, and a considerable increase in serum ALT (25.13 ± 16.90 *vs* 9.56 ± 1.99, $P < 0.01$) and AST levels (18.01 ± 10.00 *vs* 11.33 ± 4.17, $P < 0.05$) compared with control. Moreover, HL alone induced hepatocyte apoptosis, which was determined by increased TUNEL-positive cells (4.47 ± 0.45 *vs* 1.5 ± 0.22, $P < 0.01$), higher caspase-3 and lower bcl-2 expression. Interestingly, compared with those in control, HL or I/R groups, massive increases of serum ALT (93.62 ± 24.00 *vs* 9.56 ± 1.99, 25.13 ± 16.90 or 12.93 ± 6.14, $P < 0.01$) and AST (82.32 ± 26.92 *vs* 11.33 ± 4.17, 18.01 ± 10.00 or 14.00 ± 6.19, $P < 0.01$) levels in HL+I/R group were observed suggesting severe liver damage, which was confirmed by liver histology. In addition, HL combined with I/R also caused significantly increased hepatocyte apoptosis, as evidenced by increased TUNEL-positive cells (6.20 ± 0.29 *vs* 1.5 ± 0.22, 4.47 ± 0.45 or 1.97 ± 0.47, $P < 0.01$), elevated expression of caspase-3 and lower expression of bcl-2. Furthermore, when compared to HL or I/R alone, HL plus I/R enhanced serum TNF-α, IL-1, liver mitochondrial MDA and Ca^{2+} levels, suppressed SOD and GSH-Px in liver mitochondria, and markedly up-regulated the activity (11.76 ± 2.36 *vs* 4.77 ± 2.31 or 3.11 ± 1.35, $P < 0.01$) and expression (3.24 ± 0.38 *vs* 1.98 ± 0.88 or 1.72 ± 0.58, $P < 0.01$) of CYP2E1 in liver.

CONCLUSION: The coexistence of HL and acute cerebral I/R induces severe liver damage, suggesting that cerebral ischemic stroke would exaggerate the damage of liver caused by HL. This effect is possibly due to en-

hanced CYP2E1 induction which further promotes oxidative damage, inflammation and hepatocyte apoptosis.

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Key words: Hyperlipidemia; High-fat diet; Cerebral ischemia/reperfusion; Liver damage; Hepatocyte apoptosis; Cytochrome P450 2E1

Peer reviewer: Ferenc Sipos, MD, PhD, Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi u. 46., Budapest 1088, Hungary

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INTRODUCTION

The history of hyperlipidemia (HL), an increasing public health problem, is positively associated with acute cerebrovascular diseases (ACD) such as ischemic stroke[1]. Although HL *per se* is not a direct predictor of ACD, the condition with high plasma levels of inflammatory-sensitive proteins associated with HL links to the increased incidence of stroke[2]. Positive correlations between excess lipid levels and the promotion of inflammation have been documented[2]. Moreover, it has been widely accepted that oxidative stress, an early event in the evolution of HL[3], also plays an essential role in the pathophysiology of ischemic stroke[4]. So it can be speculated that HL, as a highly prevalent risk factor for ACD, frequently accompanies ACD.

In addition, HL is also a major risk factor for liver disease[5]. The spectrum of HL-induced liver disease, also known as non-alcoholic fatty liver disease (NAFLD), ranges from "simple" steatosis to non-alcoholic steatohepatitis, which is characterized by oxidative stress, inflammation, and liver damage as well as fat deposition[6]. Of interest is that one of the main complications of the patient with ACD is the risk of secondary liver dysfunction. Ischemic stroke will further trigger the activation of inflammatory cascades leading to systemic inflammatory response[7]. In the systemic inflammatory environment developed by circulating reactive oxygen species (ROS) and pro-inflammatory cytokines after ACD, nonneurological organs, including liver, would be impaired leading to multiple organ dysfunction syndromes (MODS). The incidence of MODS in patients with ACD was reported as 11.5%, the mortality rate was 40.3%[8]. It is noteworthy that one of the major systemic complications of ACD is liver dysfunction, the presence of which is associated with mortality and overall poor outcome[9]. Clinical studies in China have showed, in patients of ACD with MODS, 26.83%[8] or 15.5%[10] had abnormal liver function. Therefore, HL or ACD is respectively known to be the independent risk factor for liver injury. It is thus reasonable to hypothesize that the coexistence of HL and ACD may contribute to more severe liver dysfunction.

As mentioned above, the pathological changes in liver caused by either HL or cerebral ischemia are associated with inflammation and oxidative stress. Inflammatory response and even apoptosis could be triggered by oxidative stress[11], which may be a key mechanism of liver injury induced by HL or cerebral ischemia. The source of oxidative stress is controversial but may result in part from over-expression of the prooxidant enzyme cytochrome P450 2E1 (CYP2E1), that has been reported to occur in human and experimental NAFLD[12]. As a major microsomal source of ROS, CYP2E1 mainly located in the endoplasmic reticulum and played a significant role in the biotransformation of fatty acid oxidation. ROS overproduction induces oxidative damage to liver DNA and contributes to hepatocellular injury[13]. The expression of CYP2E1 in liver can be enhanced in rats treated with high-fat diet[14]. In addition, brain injury or inflammation has influence on some liver cytochrome P450 enzymes[15]. But the effect of HL together with ACD on CYP2E1 is still unclear.

In this study, we hypothesized that HL or cerebral ischemia/reperfusion (I/R) injury may directly induce a series of changes leading to a pro-oxidant and pro-inflammatory state, which may finally result in the onset of liver injury, and the coexistence of HL and cerebral I/R injury may cause much more severe changes and the exacerbation of liver injury. To verify this assumption, a repeatable experimental hyperlipemic rat model with cerebral I/R injury was established to investigate the correlation of HL and/or acute cerebral I/R injury on liver damage, and further examine the effects of HL and/or I/R on hepatocyte apoptosis, CYP2E1, oxidative/antioxidant system and inflammatory process to understand the mechanism of liver damage.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats, weighing 200-250 g, were obtained from Hubei Laboratory Animals Center for Medical Science and Research (No. 00006231). The animals were allowed to acclimatize for 1 wk while being maintained at a room temperature of 22 ± 2 ℃ on a 12 h light/dark cycle with free access to standard rodent chow food and water (Standard sustain feed, from Hubei Laboratory Animals Center for Medical Science and Research, Wuhan, China). Housing facilities and all experimental protocols were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animals Care and Use Committee of Wuhan University of Science and Technology Medical College which adopts the guideline

Table 1 The examined animal groups

Group	Administration
Control	Rats given normal diet (control) for 18 wk and without I/R
HL	HL rats given high-fat diet for 18 wk and without I/R
I/R	Rats given normal diet (control) for 18 wk and with I/R
HL+I/R	HL rats given high-fat diet for 18 wk and with I/R

HL: Hyperlipidemia; I/R: Ischemia/reperfusion.

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Experiment design

The rats were fed either a standard low-fat rat chow diet (control diet) or a high-fat diet (HFD, 1% cholesterol, 10% lard, 20% sugar, 5% egg yolk, 0.2% bile salts and 63.8% standard chow, finally analyzed by the Laboratory Animal Centre of Hubei). After 18 wk of HFD, blood was withdrawn from the tail vein of overnight fasted rats for confirming HL. Serum triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were detected by Beckman Coulter synchron LX20 autoanalyzer (Beckman Coulter Inc., United States). The animals with serum TC level > 3.87 mmol/L were considered HL and included in the study[16]. Subsequently, the control diet group and HL group were respectively divided into two subgroups treated with or without cerebral I/R (Table 1). After HL was confirmed, the rats in I/R or HL+I/R group were subjected to middle cerebral artery occlusion (MCAO)[17] for 2 h and followed by 24 h reperfusion to induce focal cerebral I/R injury. As the sham control, rats in control or HL groups underwent the same surgical procedure as in I/R or HL+I/R group without inserting the nylon thread.

Behavioral score and brain infarct volume were evaluated at 24 h after reperfusion by researchers and another assistant who was not involved in this experiment. The neurological deficits were scored on a 5-point scale based on the report of Longa *et al*[18]. All animals were sacrificed after the evaluation of behavioral scores. The area of cerebral infarction was quantified with TTC (2, 3, 5-triphenyltetrazolium chloride) staining as previous described[13].

Measurement of liver function and serum pro-inflammatory cytokines

Blood samples were obtained from the common carotid artery to detect serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) when rats were sacrificed at 24 h after reperfusion. The levels of serum ALT and AST were measured using corresponding kits (Jiancheng, Nanjing, China) to assess the liver function. Serum TNF-α and IL-1 levels were measured by radioimmunoassay kits (Albert Poole Biotechnology, Beijing, China).

Histological and immunohistochemical examination of liver

Harvested livers were placed into 10% formalin immediately after excision, and immersed for 24 h. Liver specimens were then embedded in paraffin, and sections were cut at 5 μm. Sections were stained for: (1) Hematoxylin and eosin (HE); (2) Apoptosis was detected by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method using an *in situ* apoptosis detection kit (Boster Biological Engineering, Wuhan, China) as previously described[19]. The number of cells with TUNEL-positive nuclei was counted from 20 randomly selected fields at × 200 magnification per liver sample. Results were expressed as the mean number of TUNEL-positive apoptotic hepatocytes per microscopic field; and (3) Immunohistological analysis of CYP2E1, caspase-3, bcl-2 expression in paraffin wax-embedded liver sections were performed using a standard peroxidase-antiperoxidase technique as described previously[20], using a CYP2E1 rabbit anti-mouse polyclonal antibody (Abcam Inc, United Kingdom) or mouse monoclonal antibodies against caspase-3 and bcl-2 (Santa Cruz, CA, United States) at a 1:150 dilution, with a biotinylated goat anti-rabbit or goat anti-mouse antibody (Santa Cruz, CA, United States) as the secondary antibody. Brown colour in the cytoplasm of the hepatocytes was evaluated as positive staining.

Determination of antioxidant enzymes activities, MDA and Ca^{2+} content in liver mitochondria and microsomal CYP2E1-dependent aniline hydroxylase activity

Liver subcellular fractions were prepared by differential centrifugation according to Khemawoot *et al*[21] with slight modifications. Livers were homogenized (20% w/v) with phosphate buffer (50 mmol/L K_2HPO_4 containing 0.1 mmol/L EDTA, pH 7.4). The homogenate was centrifuged at 200 *g* for 10 min at 4 ℃, and then the supernatant was recentrifuged at 9000 *g* for 20 min. The mitochondrial pellet was collected, resuspended in 0.25 mol/L sucrose solution, and stored at -80 ℃ until detection of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and Ca^{2+} levels using the corresponding kits (Jiancheng, Nanjing, China) respectively.

Then the supernatant was recentrifuged at 105 000 × *g* for 60 min. The microsomal pellet was washed, resuspended in 0.25 mol/L sucrose solution, and stored at -80 ℃ until determination of CYP2E1 activity and expression. Microsomal protein concentration was measured according to the method of Lowry *et al*[22]. Microsomal hydroxylase activity of the CYP2E1-containing enzyme was measured with aniline as the substrate according to the procedure described previously[23].

Western blotting of liver caspase-3, bcl-2 and microsomal CYP2E1

Proteins of liver homogenate or microsome were separated by SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane by electroblotting.

Table 2 Blood lipids in different groups after high-fat diet treatment for 18 wk and before middle cerebral artery occlusion (mean ± SD, mmol/L)

Group	*n*	TC	TG	HDL-C	LDL-C
Control	10	1.69 ± 0.14	0.21 ± 0.08	1.14 ± 0.05	0.40 ± 0.10
HL	6	5.11 ± 0.55[b,e]	0.84 ± 0.20[a,f]	0.68 ± 0.15[a,f]	1.72 ± 0.52[a,e]
I/R	10	1.57 ± 0.19	0.19 ± 0.02	1.21 ± 0.16	0.36 ± 0.10
HL+I/R	10	5.33 ± 0.44[b,e]	0.79 ± 0.17[a,f]	0.70 ± 0.19[a,f]	1.64 ± 0.48[a,e]

HL: Hyperlipidemia; I/R: Ischemia/reperfusion; TC: Total cholesterol; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [e]$P < 0.05$, [f]$P < 0.01$ *vs* I/R group.

Membrane was blocked overnight and then incubated for 2 h with a 1:1000 dilution of a rabbit polyclonal anti-rat CYP2E1 antibody (Abcam Inc, United Kingdom) or a rabbit polyclonal anti-mouse, caspase-3, bcl-2 antibody (Santa Cruz, CA, United States). After incubation with the secondary antibody, proteins were detected with an electrochemiluminescence chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, United Kingdom), and scanned with a Typhoon 9200 scanner (Amersham Biosciences Europe GmbH, Freiburg, Germany). The amount of protein expression was corrected by the amount of β-actin in the same sample.

Statistical analysis

All data are expressed as mean ± SD. Statistical analysis was performed using Student's *t* test. Probability values less than 0.05 ($P < 0.05$) were considered statistically significant.

RESULTS

Evaluation of animal model

After 18 wk of HFD and prior to ischemia, serum levels of TC, TG and LDL-C in HL or HL+I/R group were significantly higher than those in control or I/R group, whereas HDL-C was lower ($P < 0.05$ or $P < 0.01$) (Table 2). There was no significant difference between the HL group and the HL+I/R group.

At 24 h after reperfusion, rats in the I/R or HL+I/R groups that were successfully operated with MCAO and reperfusion appeared to have a significantly higher neurological deficit score and brain infarct volume than those in the control or HL group ($P < 0.01$) (Table 3). There was no significant difference between the I/R group and the HL+I/R group.

These results strongly suggested that the animal model of HL combined with I/R was established successfully.

Liver damage caused by HL plus I/R

Plasma ALT and AST levels are common biomarkers for liver damage[24]. As compared to the control group, plasma activities of ALT and AST were elevated in the HL group ($P < 0.05$; $P < 0.01$), but not affected significantly by I/R alone; marked changes in serum ALT and AST activities suggesting hepatic damage were shown in the HL+I/R group ($P < 0.01$). The most severe liver damage was found in the HL+I/R group compared to HL or I/R alone ($P < 0.01$) (Table 3).

Table 3 Brain infarct volume and serum alanine aminotransferase and aspartate aminotransferase in different groups after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion (mean ± SD)

Group	*n*	Neurological deficit score	Infarct volume (mm^3)	ALT (U)	AST (U)
Control	10	0	0	9.56 ± 1.99	11.33 ± 4.17
HL	6	0	0	25.13 ± 16.90[b]	18.01 ± 10.00[a]
I/R	7	3.25 ± 0.36[b,d]	33.4 ± 4.16[b,d]	12.93 ± 6.14	14.00 ± 6.19
HL+I/R	6	3.53 ± 0.45[b,d]	34.5 ± 6.23[b,d]	93.62 ± 24.00[b,d,f]	82.32 ± 26.92[b,d,f]

HL: Hyperlipidemia; I/R: Ischemia/reperfusion; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* HL group; [f]$P < 0.01$ *vs* I/R group.

To analyze the extent of liver injury, liver sections were stained with HE (Figure 1A). No apparent damage was found in liver sections from the control or I/R group. Hepatocytes of the HL group showed fatty infiltration changes, light vacuolar change, scattered inflammatory cells and mild hepatocyte necrosis. However, extensive damage was detected in liver sections from HL+I/R rats. The affected livers displayed remarkable fatty accumulation, infiltration of a mixed population of inflammatory cells, hepatocyte ballooning degeneration and focal necrosis. These changes were more prominent when compared to HL or I/R alone.

Hepatocyte apoptosis caused by HL plus I/R

We assessed hepatocyte apoptosis using the TUNEL assay. TUNEL-positive cells appeared occasionally in liver sections of control or I/R groups, diffused widely in the HL group, and were most frequently observed in the HL+I/R group (Figure 1B). The number of TUNEL-positive apoptotic hepatocytes (6.2 ± 0.29 apoptotic cells/× 200 field) in the HL+I/R group was significantly greater than those in the control group (1.5 ± 0.22 apoptotic cells/× 200 field), HL group (4.47 ± 0.45 apoptotic cells/× 200 field), and I/R group (1.97 ± 0.47 apoptotic cells/× 200 field) ($P < 0.01$).

Encouraged by the severe liver damage and hepatocyte apoptosis caused by HL plus I/R, we further investigated whether the CYP2E1, oxidative/antioxidant system and pro-inflammatory cytokines are involved in this pathological condition.

Induction of hepatic CYP2E1 by HL plus I/R

The activities of CYP2E1-dependent aniline hydroxylase activity in the microsome, where CYP2E1 is mainly located in liver, were higher in the HL or the I/R group ($P < 0.01$; $P < 0.05$ *vs* control), and increased consider-

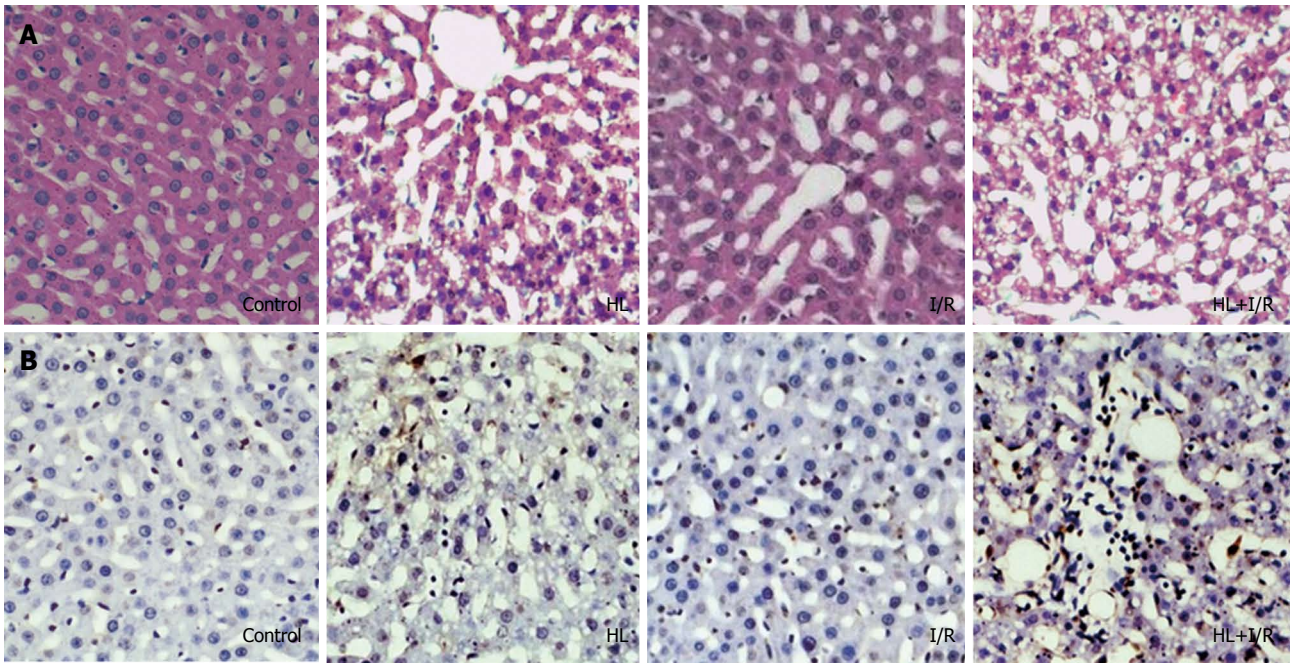


Figure 1 Histology and in situ apoptosis of liver sections from rats after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion. A: HE staining showed liver damage in the hyperlipemia (HL) group and especially in the HL+ ischemia/reperfusion (I/R) group (× 200); B: Transferase dUTP nick-end labeling (TUNEL) staining showed TUNEL-positive apoptotic hepatocytes in the HL+I/R group are significantly greater than those in control, HL or I/R groups (× 200). Slides are representative of 6-10 animals per group.

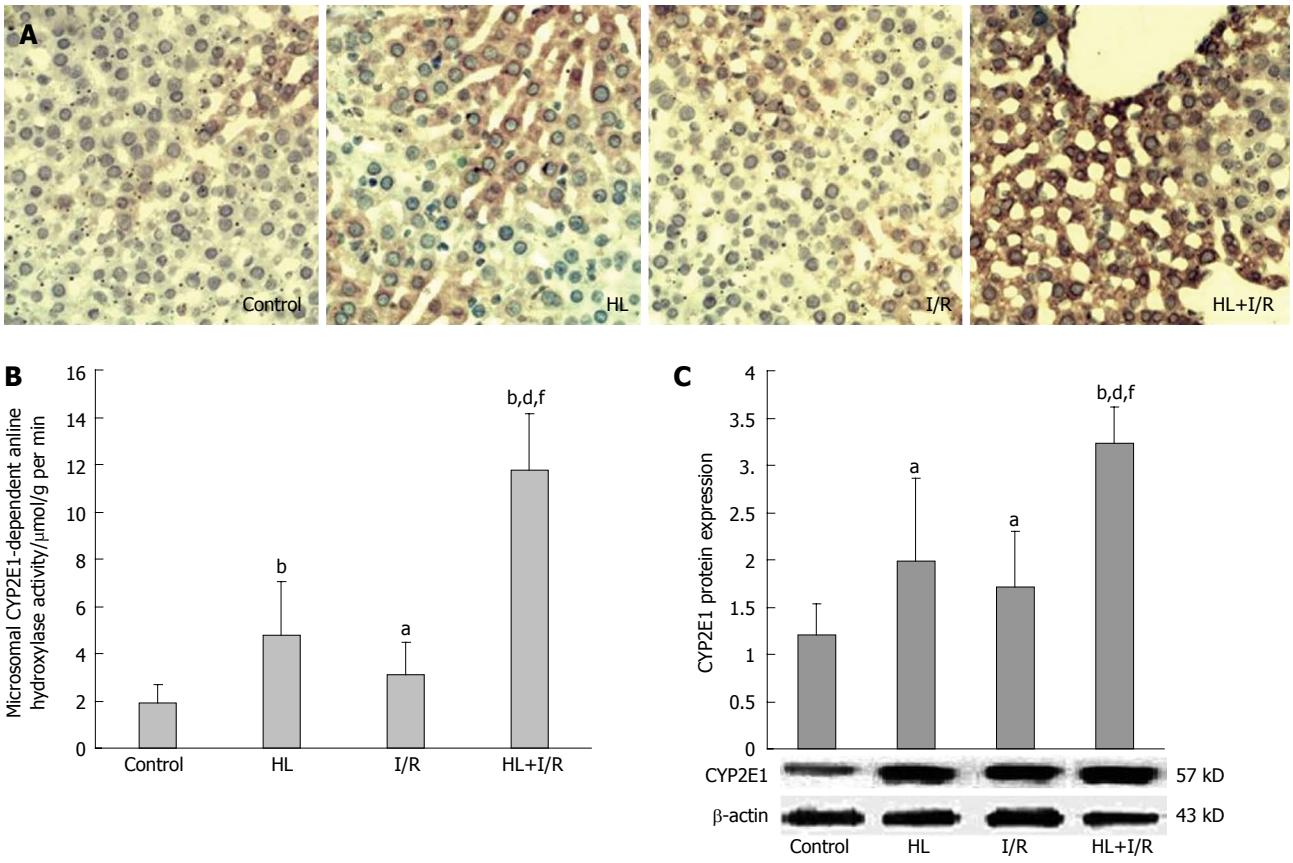


Figure 2 Effect of hyperlipemia plus ischemia/reperfusion on the activity and expression of CYP2E1 in the liver of rats after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion. A: Hepatocytes in the hyperlipemia (HL) + ischemia/reperfusion (I/R) group showed increased cytochrome P450 2E1 (CYP2E1) expression examined by immunohistochemical staining (× 200) than those in other groups. Slides are representative of 6-10 animals per group; B: Microsomal hydroxylase activity of the CYP2E1-containing enzyme measured with aniline as the substrate was much higher in the HL+I/R group; C: HL+I/R enhanced the expression level of CYP2E1 protein in liver microsomes, which was confirmed by Western blotting. Values are the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* HL group; $^{f}P < 0.01$ *vs* I/R group.

Table 4 Levels of antioxidant enzymes, malondialdehyde and Ca^{2+} in liver mitochondria of different groups after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion (mean ± SD)

Group	*n*	SOD (U/μg protein)	GSH-Px (nmol/g protein per min)	MDA (mmol/g protein)	Ca^{2+} (μmol/g protein)
Control	10	244.93 ± 33.70	2172.26 ± 241.34	7.02 ± 2.58	1.329 ± 0.279
HL	6	100.59 ± 40.72[b]	937.57 ± 228.68[b]	21.84 ± 6.01[b]	2.552 ± 0.317[b]
I/R	7	172.15 ± 57.31[a]	1762.65 ± 409.18	12.58 ± 5.34	2.180 ± 0.431[b]
HL+I/R	6	59.01 ± 21.48[b,c,f]	486.41 ± 230.52[b,d,f]	47.27 ± 7.11[b,c,f]	3.136 ± 0.517[b,c,f]

HL: Hyperlipidemia; I/R: Ischemia/reperfusion; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase; MDA: Malondialdehyde. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* HL group; [f]$P < 0.01$ *vs* I/R group.

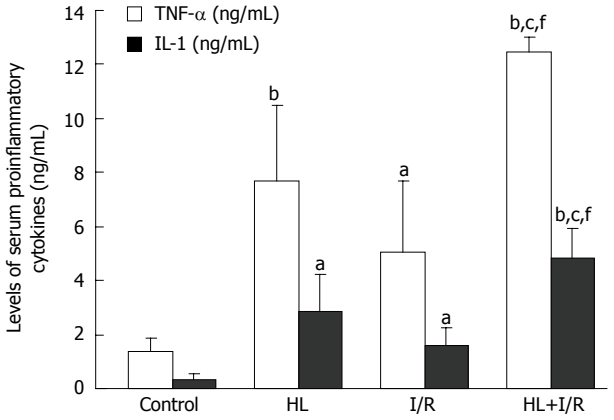


Figure 3 Effect of hyperlipemia + ischemia/reperfusion on the level of serum pro-inflammatory cytokines after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion. Serum pro-inflammatory cytokines in the hyperlipemia (HL) + ischemia/reperfusion (I/R) group were significantly higher compared to those in other groups. A: Tumor necrosis factor (TNF)-α; B: Interleukin (IL)-1. Values are the mean ± SD. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [c]$P < 0.05$ *vs* HL group; [f]$P < 0.01$ *vs* I/R group.

ably especially in the HL+I/R group ($P < 0.01$ *vs* control, HL or I/R groups) (Figure 2B). To understand the basis of aniline hydroxylase activity in rat liver microsome, we carried out immunohistochemistry and Western blotting analysis of CYP2E1. Immunohistochemistrical staining for hepatic CYP2E1 was occasionally detectable in the control group, but increased in the HL or I/R groups. In the HL+I/R group, strong and dense CYP2E1 immunoreactivity was observed, which paralleled the improvement in histological severity of liver damage (Figure 2A). Similar effects were observed in Western blotting. The protein expression of microsomal CYP2E1 was induced by HL ($P < 0.05$ *vs* control) and I/R ($P < 0.05$ *vs* control), and highly enhanced by HL+I/R ($P < 0.01$ *vs* control, HL or I/R group) (Figure 2C).

Effect of HL plus I/R on the levels of antioxidant enzymes, MDA and Ca^{2+} in liver mitochondria

The activities of antioxidant enzymes, including SOD and GSH-Px, fell dramatically in the HL+I/R group ($P < 0.01$ *vs* control, $P < 0.05$ or $P < 0.01$ *vs* HL alone, $P < 0.01$ *vs* I/R alone). SOD activities of the HL, I/R and HL+I/R groups were, respectively, 41.1%, 70.3% and 24.1% of control level; GSH-Px activities were 43.2%, 81.1% and 22.4% of control level, respectively (Table 4).

MDA and Ca^{2+} levels in liver mitochondria were higher in the HL group than the control group ($P < 0.01$). Liver mitochondrial Ca^{2+} levels also increased in the I/R group ($P < 0.01$ *vs* control). In the HL+I/R group, MDA and Ca^{2+} levels were most significantly increased compared with all other groups ($P < 0.01$ *vs* control or I/R group, $P < 0.05$ *vs* HL group) (Table 4).

Effect of HL plus I/R on the level of serum pro-inflammatory cytokines (TNF-α and IL-1)

The levels of serum TNF-α and IL-1 in HL, I/R or HL+I/R groups were significantly higher compared to those in the control group ($P < 0.01$; $P < 0.05$). When compared with HL or I/R alone, the increase of these two pro-inflammatory cytokines in the HL+I/R group also had statistical significance ($P < 0.05$ *vs* HL, $P < 0.01$ *vs* I/R group) (Figure 3).

Effect of HL plus I/R on the expressions of caspase-3 and bcl-2

We further investigated the expression levels of two key apoptosis-related genes- caspase-3 and bcl-2-in liver tissue, which were determined by immunohistochemistry and Western blotting. Immunohistochemistry assay showed protein expression of caspase-3 (Figure 4A) in livers of HL and HL+I/R rats was markedly increased, and the anti-apoptosis gene bcl-2 (Figure 4B) was dramatically decreased. These findings were confirmed by Western blotting, which showed increased expression of caspase-3 (Figure 4C) and decreased expression of bcl-2 (Figure 4D), especially in the HL+I/R group.

DISCUSSION

Dyslipidemia is usually responsible for liver disease including cirrhosis, hepatocellular carcinoma and liver failure[5]. It has also been reported that "high-fat diet" can induce lipid accumulation in rat liver by nutritional intervention[25]. In agreement with previous work[26-28], our study also demonstrated that HL alone induced by HFD for 18 wk resulted in considerable increases in serum ALT and AST levels, marked pathological changes in the liver, increased TUNEL-positive cells, higher caspase-3 and lower bcl-2 expression, suggesting liver damage and hepatocyte apoptosis. Oxidative stress and subsequent

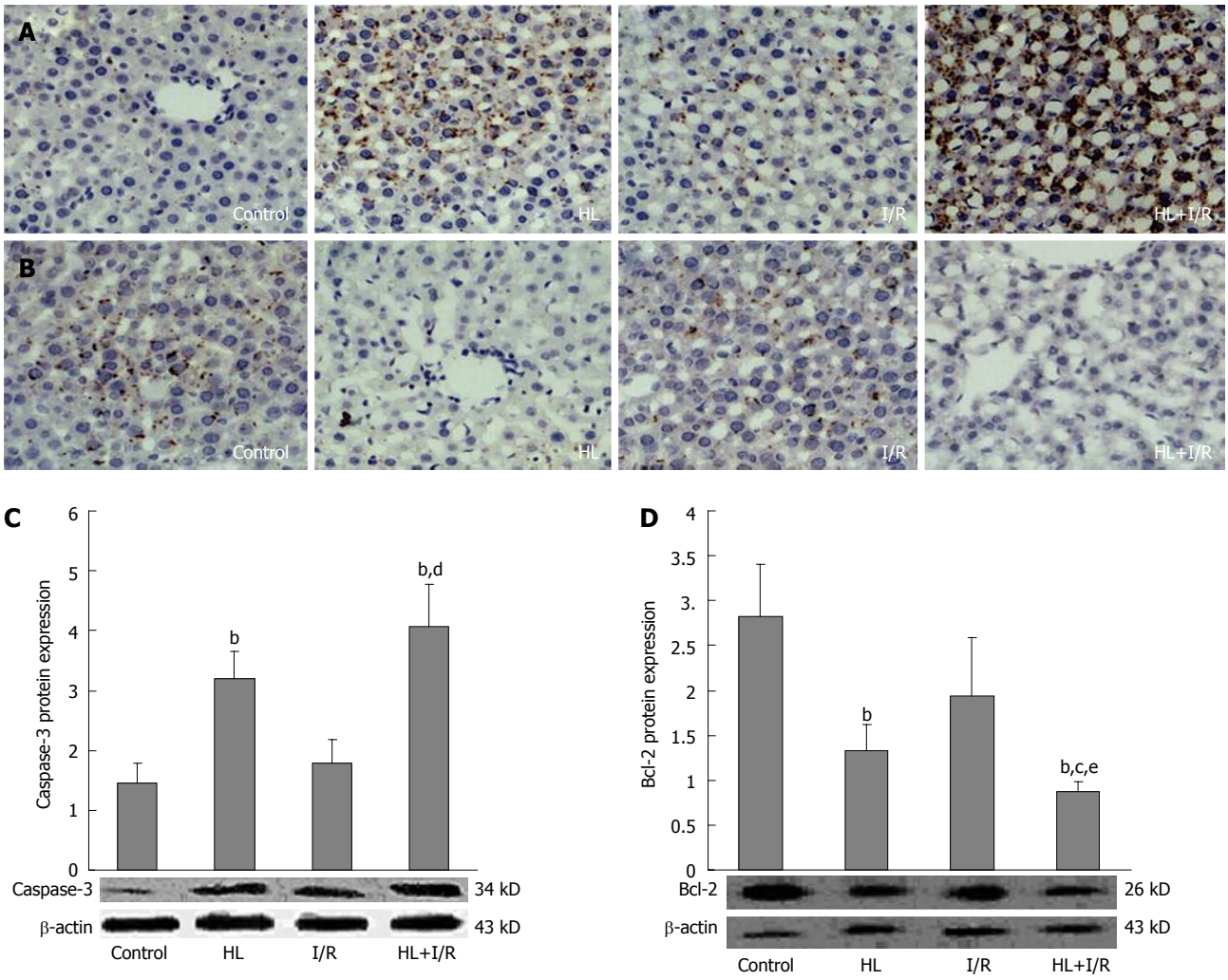


Figure 4 Expressions of bcl-2 and caspase-3 protein in liver after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion. Liver sections from different groups were immunohistochemically stained to assay the expression of caspase-3 and bcl-2 (× 200). A: Protein expression of caspase-3 was markedly elevated in the hyperlipemia (HL) + ischemia/reperfusion (I/R) group; B: Protein expression of bcl-2 was dramatically decreased in the HL+I/R group. C, D: Slides are representative of 6-10 animals per group. Liver homogenates were prepared, and then the expression levels of caspase-3 (C) and bcl-2 (D) proteins in liver were confirmed by Western blotting. Values are the mean ± SD. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* HL group; $^{e}P < 0.05$ *vs* I/R group.

lipid peroxidation, together with the production of pro-inflammatory cytokines have been shown to play important roles in the pathogenesis of nonalcoholic fatty liver disease induced by HFD[29,30], which were also confirmed in our current experiment.

Besides liver damage caused by long-term HFD, our findings are consistent with the literature stating ischemic stroke *per se* is possibly associated with liver dysfunction[31]. Although no significant change in serum ALT and AST levels, histologic examination of liver, the number of TUNEL-positive cells, caspase-3 and bcl-2 expression in liver was found, systemic inflammation and the imbalance in oxidant production and antioxidant defense systems, which are strongly associated with the development of MODS due to I/R, were indeed discovered in the I/R group. Actually, circulating pro-inflammatory cytokines, for example, TNF-α and IL-1, and systemic oxidative stress would influence the nonneurological organs including liver[32].

Taken together, HL *per se* induced systemic inflammation and oxidative stress, and eventually caused hepatocyte apoptosis and liver damage, while I/R *per se* also induced systemic inflammation and oxidative stress but did not cause obvious hepatocyte apoptosis and liver damage.

Most interestingly, we demonstrated that, as expected, the combined action of HL and I/R brought about an abrupt reduction in hepatic function with enhanced hepatocyte apoptosis, which is much worse than the damage caused by I/R or HL alone. The incidence of ischemic stroke increases dramatically with advancing age. Due to aging, many patients with cerebral ischemia also suffer from HL, which is another common geriatric disease. It has also been reported that HL, as a cardiovascular risk factor, would lead to intracranial artery atherosclerosis or blockage, aggravate cerebral infarction, and exacerbate the deleterious effects of I/R on the brain[33]. In our

study, we found the coexistence of HL and cerebral I/R injury would be more dangerous because it induces more severe visceral dysfunction i.e., liver damage than HL alone. This kind of systemic complication may be fatal subsequently.

This liver damage secondary to the coexistence of HL and cerebral I/R injury can be explained readily by the high levels of circulating pro-inflammatory cytokines and oxidative stress, which possibly result from the synergy of systemic inflammatory and oxidative milieu induced by HL *per se* and those induced by I/R *per se*. In support of these concepts, we showed that, compared with I/R or HL alone, enhanced CYP2E1 induction, an increase in serum TNF-α, IL-1, liver mitochondrial MDA, and a decrease in SOD and GSH-Px in liver mitochondria were also measured in the HL+I/R group, which indicated that the coexistence of HL with cerebral I/R injury is associated with increased systemic inflammation and oxidative stress.

A significant change in CYP2E1 should be noted in the liver of HL rats with cerebral I/R injury. CYP2E1, sometimes mediating liver damage through oxidative stress[34,35], is a major adaptive system in the CYP superfamily. CYP2E1 has adjustability, and is inducible under a variety of physiological or pathophysiological conditions. It has been demonstrated that CYP2E1 levels increased in obese rats by feeding with an energy-dense diet[14] and responded quickly to acute stress, such as hypoxia, cold or hunger[36]. In fact, acute cerebral I/R injury also induces an acute stress condition in animals. In the present study, we have corroborated the induction of CYP2E1 by HL *per se* or I/R *per se*, especially I/R combined with HL, as an increase in hepatic CYP2E1 activity and protein expression was found in the HL and I/R groups, and especially in the HL+I/R group. CYP2E1 is known to be a key indicator of lipid peroxidation, and a significant source of ROS[12] contributing to hepatocellular injury. Enhanced CYP2E1 induction oxidizes mitochondrial DNA, proteins and lipids, and triggers hepatic TNF-α formation by activating nuclear factor-κB, which further increases mitochondrial ROS formation. ROS overproduction leads to inflammatory recruitment and apoptosis from oxidative stress[11], and finally exacerbates the disease.

Consistent with the change in MDA, we also revealed that mitochondrial Ca^{2+} content increased in the HL+I/R group. An imbalance of Ca^{2+} homeostasis is thought to be the "common pathway" of hepatocyte damage. Mitochondrial and cellular Ca^{2+} overload facilitates lipid peroxidation, which attenuates the oxidative phosphorylation of the mitochondrion resulting in damage to the mitochondrial structure and function and then the reduction of ATP synthesis. On the other hand, lipid peroxidation will in turn exacerbate the Ca^{2+} overload by acting on the membrane structure[37]. Accordingly, mitochondrial Ca^{2+} overload of hepatocytes is closely relevant to lipid peroxidation, and both are likely to be involved in hepatic injury after HL+I/R.

Moreover, we have evaluated, by immunohistochemistry and Western blotting analysis, two key mediators of apoptosis (caspase-3 and bcl-2), which appear to be adjusted by ROS and cytokines, including TNF-α and IL-1[32,38]. Increased protein expression of key pro-apoptotic factor caspase-3 and markedly down-regulated anti-apoptotic bcl-2 detected in livers of HL rats with cerebral I/R injury in our study which resulted in hepatocyte apoptosis and liver damage.

In summary, based on an experimental HL rat model with cerebral I/R, the present study revealed that the coexistence of HL and acute cerebral I/R induced severe liver damage. These findings suggested cerebral ischemic stroke would exaggerate the damage of liver caused by HL. So it can be reckoned that patients of HL combined with cerebral I/R injury have a higher risk of liver damage compared with patients with HL or cerebral I/R injury alone, the mechanism of which is possibly due to enhanced CYP2E1 induction, which can further increase mitochondrial ROS formation, oxidative damage, inflammation and apoptosis with increased protein expression of key pro-apoptotic factor caspase-3, and markedly down-regulated anti-apoptotic bcl-2. Therefore, special precautions against subsequent liver injury are required after the burst of cerebral ischemic stroke in patients with HL. It seems that the therapeutic approaches to inhibit CYP2E1 activation, rebalance the oxidative/antioxidant system, and suppress inflammation or apoptosis might be efficacious in preventing the liver damage induced by HL plus cerebral I/R injury.

COMMENTS

Background

The history of hyperlipidemia (HL), an increasingly public health problem, is positively associated with acute cerebrovascular diseases (ACD) such as ischemic stroke. Moreover, HL is also a major risk factor for liver disease. One of the main complications of the patient with ACD is the risk of secondary liver dysfunction. It is thus reasonable to hypothesize that the coexistence of HL and ACD may contribute to more severe liver dysfunction.

Research frontiers

The pathological changes in liver caused by either HL or cerebral ischemia are associated with inflammation and oxidative stress. An inflammatory response and even apoptosis could be triggered by oxidative stress, which may be a key mechanism of liver injury induced by HL or cerebral ischemia. The source of oxidative stress is controversial but may result in part from over-expression of the prooxidant enzyme cytochrome P450 2E1 (CYP2E1). CYP2E1 has adjustability, and is inducible under a variety of physiological or pathophysiological conditions. It has been demonstrated that CYP2E1 levels are enhanced in obese rats by feeding with an energy-dense diet and respond quickly to acute stress, such as hypoxia, cold or hunger.

Innovations and breakthroughs

HL or ACD have been known to be independent risk factors for liver injury. In this study, the authors developed a repeatable experimental hyperlipemic rat model with acute cerebral ischemia/reperfusion injury, and investigated the correlation of HL and/or acute cerebral ischemia/reperfusion injury on liver damage, and further examine the effects of HL and/or ischemia/reperfusion on hepatocyte apoptosis, CYP2E1, the oxidative/antioxidant system and inflammatory process to understand the mechanism of liver damage.

Applications

The study results suggest that cerebral ischemic stroke would exaggerate the

damage of liver caused by HL. So it can be reckoned that patients with HL combined with acute cerebral ischemia/reperfusion injury have a higher risk of liver damage compared with patients with HL or cerebral ischemia/reperfusion injury alone, the mechanism of which is possibly due to enhanced CYP2E1 induction, which can further increase mitochondrial reactive oxygen species formation, oxidative damage, inflammation and apoptosis with increased protein expression of key pro-apoptotic factor caspase-3, and markedly down-regulated anti-apoptotic bcl-2. Therefore, special precautions against subsequent liver injury are required after the burst of cerebral ischemic stroke in patients with HL. It seems that therapeutic approaches to inhibit CYP2E1 activation, rebalance the oxidative/antioxidant system, suppress inflammation or apoptosis might be efficacious in prevention and treatment of liver damage induced by HL plus cerebral ischemia/reperfusion injury.

Peer review

The topic is of great clinical importance as the prevalence and incidence of hyperlipidemia and related cerebrovascular diseases as well as non-alcoholic fatty liver disease are increasing in industrialized countries; hence the associated financial problems is a major point to cope with. The rat model based study is well designed and the results are significant.

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BRIEF ARTICLE

Does gemcitabine-based combination therapy improve the prognosis of unresectable pancreatic cancer?

Chen Sun, Daniel Ansari, Roland Andersson, De-Quan Wu

Chen Sun, De-Quan Wu, Department of Surgery, Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang Province, China
Chen Sun, Daniel Ansari, Roland Andersson, Department of Surgery, Clinical Sciences Lund, Lund University, Skåne University Hospital, SE-22185 Lund, Sweden
Author contributions: Sun C and Ansari D performed the majority of the study and wrote the manuscript; Andersson R and Wu DQ conceived the study and finalized the revision; all authors read and approved the final manuscript.
Correspondence to: De-Quan Wu, MD, PhD, Department of Surgery, Second Affiliated Hospital of Harbin Medical University, Xuefu Road, Harbin 150086, Heilongjiang Province, China. dqwu56@163.com
Telephone: +86-451-86605535 Fax: +86-451-82524051
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Abstract

AIM: To assess whether gemcitabine-based combination therapy improves the prognosis of unresectable pancreatic cancer compared with gemcitabine treatment alone.

METHODS: A quantitative up-to-date meta-analysis was undertaken to investigate the efficacy of gemcitabine-based combination treatment compared with gemcitabine monotherapy in locally advanced or metastatic pancreatic cancer. Inclusion was limited to high-quality randomized clinical trials.

RESULTS: Twenty-six studies were included in the present analysis, with a total of 8808 patients recruited. The studies were divided into four subgroups based on the different kinds of cytotoxic agents, including platinum, fluoropyrimidine, camptothecin and targeted agents. Patients treated with gemcitabine monotherapy had significantly lower objective response rate [risk ratio (RR), 0.72; 95% confidence interval (CI): 0.63-0.83; $P < 0.001$], and lower 1-year overall survival (RR, 0.90; 95%CI: 0.82-0.99; $P = 0.04$). Gemcitabine monotherapy caused fewer complications, including fewer grade 3-4 toxicities: including vomiting (RR, 0.75; 95%CI: 0.62-0.89; $P = 0.001$), diarrhea (RR, 0.66; 95%CI: 0.49-0.89; $P = 0.006$), neutropenia (RR, 0.88; 95%CI: 0.72-1.06; $P = 0.18$), anemia (RR, 0.96; 95%CI: 0.82-1.12; $P = 0.60$), and thrombocytopenia (RR, 0.76; 95%CI: 0.60-0.97; $P = 0.03$) compared with gemcitabine combination therapies.

CONCLUSION: Gemcitabine combination therapy provides a modest improvement of survival, but is associated with more toxicity compared with gemcitabine monotherapy.

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Key words: Pancreatic cancer; Gemcitabine; Combination therapy; Outcome; Meta-analysis

Peer reviewers: Itaru Endo, MD, PhD, Professor and Chairman, Department of Gastroenterological Surgery, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 2360004, Japan; Cuong D Tran, PhD, Research Fellow, Affiliate Lecturer, University of Adelaide, Gastroenterology Unit, Children, Youth and Women's Health Service, 72 King William Rd, North Adelaide, SA 5006, Australia

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INTRODUCTION

Pancreatic cancer is a highly lethal disease, and represents the fourth cause of cancer-related death in the Western

world. In the United States, the incidence of pancreatic cancer almost equals its mortality, with 43 140 estimated new cases and 36 800 deaths in 2010. Globally, the incidence of new cases is approximately 230 000 each year[1,2]. Despite advances in diagnosis, staging, and surgical management of the disease during the past decade, the 5-year survival rate in the United States, Europe, and Australia is less than 5%[3-5]. Pancreatic cancer is characterized by a rapid disease progression and highly invasive tumor phenotype, with most patients having unresectable disease at diagnosis, and chemotherapy being the only possible treatment option for these patients[6].

Gemcitabine is a pyrimidine antimetabolite and analog of deoxycytidine. It was approved by the Food and Drug Administration as a first-line treatment for patients with locally advanced (stage Ⅱ or stage Ⅲ disease when surgery is not an option) or metastatic (stage Ⅳ) cancer of the pancreas, and has been widely used during the last decade. However, because of high levels of intrinsic and acquired chemoresistance, a large number of patients do not respond to gemcitabine[7]. To improve clinical efficacy, systemically administered gemcitabine is often combined with a second cytotoxic agent, such as platinum analogs, fluoropyrimidine, or a targeted cytotoxic agent. Numerous clinical trials have aimed at proving the superiority of gemcitabine-based combination therapy over single-agent gemcitabine treatment. However, most of the results of clinical trials have important limitations, including lack of statistical power because of small study populations. Thus, there remain several areas of controversy and uncertainty concerning optimal treatment regimens.

In the present study, we undertook a quantitative up-to-date meta-analysis to investigate the efficacy of gemcitabine-based combination treatment in locally advanced or metastatic pancreatic cancer. The aim of this study was to assess whether gemcitabine-based combination therapy improves the prognosis compared with gemcitabine treatment alone and to discuss possible mechanisms.

MATERIALS AND METHODS

Literature search strategy

We carried out a comprehensive search of the literature, including PUBMED (http://www.ncbi.nlm.nih.gov/pubmed), EMBASE (http://www.embase.com/home), American Society of Clinical Oncology abstracts (http://www.asco.org), and the Cochrane Database (http://www.thecochranelibrary.com). The following keywords were used in the search: ("Gemcitabine" or "Gemzar"), and ("pancreatic cancer" or "pancreatic tumor" or "pancreatic carcinoma"), and ("clinical trial"). The deadline for a publication to be eligible for this study was November 5, 2011. In addition to the online search, references from reviews and original articles were also scanned manually to identify further trials that met the eligibility criteria. No language restrictions were applied.

Inclusion and exclusion criteria

Studies included in the analysis had to meet all of the following criteria: (1) prospective, randomized, controlled open or blinded trial; (2) patients with histologically confirmed locally advanced or metastatic pancreatic ductal adenocarcinoma; and (3) assessment of the efficacy of gemcitabine combination therapy *vs* gemcitabine alone. Non-randomized trials and quasi-randomized trials, studies of curatively aimed resection, and studies where patients had multiple cancers, were excluded to avoid clinical heterogeneities between different studies.

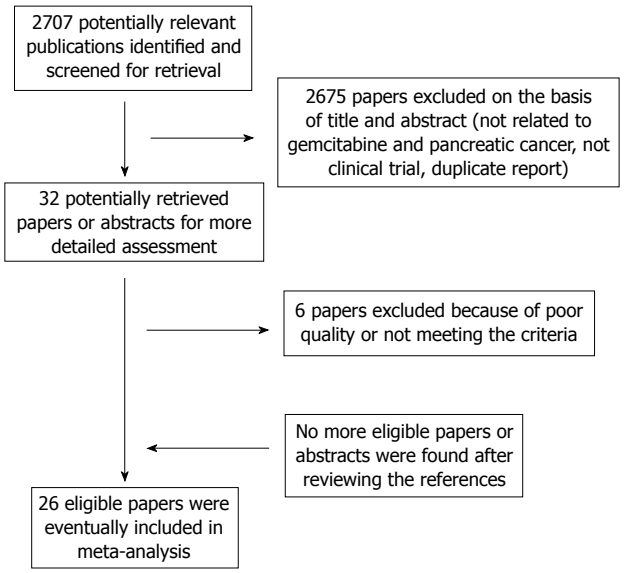


Figure 1 Flow chart of study selection.

Data extraction and quality assessment

Data extraction and quality assessment were performed independently by two reviewers (CS and DA). Any disagreements between the reviewers were discussed with a third reviewer (RA) to achieve a consensus. The data extracted from the eligible studies included: first author, year of publication, patient characteristics, intervention, and clinical outcome (toxicity, response rate, overall survival and progression-free survival). If the same trial appeared on sequential or multiple publications, the data from the most recent or comprehensive one was included.

The methodological quality of included studies was assessed using the "Jadad scale" or "Oxford quality scoring system"[8]. This tool is an evidence-based quality assessment tool. There are three items (randomization, double blinding, withdrawals and dropouts) directly related to bias reduction for assessment. Each item is given a score of 1 point for each "yes" or 0 points for each "no", and 1 additional point for appropriate randomization and double blinding. Every eligible study were assessed and given a score from 0-5.

Data analysis

The outcome measures were objective response rate [objective response rate (ORR) = complete response (CR) + partial response (PR)], as previously defined[9], 1-year overall survival (OS), median progression-free survival (PFS), median OS and toxicity. The analysis of ORR

Table 1 Characteristics of selected trials

Ref.	Intervention	*n*	Male (%)	Age (range)	Metastatic (%)	Response rate (%)	Median PFS (mo)	1-yr OS	Median OS (mo)	Quality
Louvet *et al*[15]	Gem	163	53	60.1 (22-75)	70	17.3	3.7	27.8	7.1	4
	Gem + Oxaliplatin	163	60	61.3 (35-77)	68	26.8	5.8	34.7	9.0	
Poplin *et al*[16]	Gem	275	56.4	64 (31-88)	90.2	6	2.6	16	4.9	4
	Gem + Oxaliplatin	272	45.6	63 (29-96)	89.3	9	2.7	21	5.7	
Heinemann *et al*[17]	Gem	95	61.9	66 (43-85)	78.9	9.0	3.1	24.7	6.0	4
	Gem + Cisplatin	95	65.3	64 (37-82)	80	11.5	5.3	25.3	7.5	
Colucci *et al*[18]	Gem	54	50	63 (43-75)	65	9.2	2	11	5	4
	Gem + Cisplatin	53	35	60 (33-71)	68	26.4	5	11.3	7.5	
Colucci *et al*[19]	Gem	199	56.8	63 (37-75)	82.9	10.1	3.9	34.0	8.3	4
	Gem + Cisplatin	201	62.2	63 (35-75)	84.6	11.4	3.8	30.7	7.2	
Kulke *et al*[20]	Gem	64	66	58.9 (31-81)		14	3.3	NR	6.4	4
	Gem + Cisplatin	66	56	58.9 (36-84)	100	13	4.5	NR	6.7	
	Gem + Irinotecan	64	68	60.8 (32-77)		14	4.0	NR	7.1	
Berlin *et al*[21]	Gem	162	53.7	64.3 (33-85)	90.1	5.6	2.2	NR	5.4	4
	Gem + 5-fluorouracil	160	51.8	65.8 (28-84)	89.4	6.9	3.4	NR	6.7	
Herrmann *et al*[22,24]	Gem	159	53	62 (36-84)	79	8	3.9	30	7.2	4
	Gem + Capecitabine	160	54	62 (27-83)	80	10	4.3	32	8.4	
Cunningham *et al*[23]	Gem	266	58	62 (26-83)	71	12.4	3.8	22.0	6.2	4
	Gem + Capeitabine	267	60	62 (37-82)	70	19.1	5.3	24.3	7.1	
Scheithauer *et al*[25]	Gem	42	55	66 (39-75)	100	14	4.0	37.2	8.2	4
	Gem + Capeitabine	41	66	64 (40-75)	100	17	5.1	31.8	9.5	
Costanzo *et al*[26]	Gem	49	48	64 (34-75)	73	8	3.5	18	7.75	4
	Gem + 5-fluorouracil	45	63	62 (44-75)	67	11	4.5	20	7.5	
Abou-Alfa *et al*[27]	Gem	174	57	62.3 (30-84)	78	5.7	3.8	21	6.2	4
	Gem + Exatecan	175	53	63.0 (36-85)	79	7.1	3.7	23	6.7	
Stathopoulos *et al*[28]	Gem	74	42	64 (44-83)	66	10	2.9	21.8	6.5	4
	Gem + Irinotecan	71	39	64 (31-84)	60	15	2.8	24.3	6.4	
Lima *et al*[29]	Gem	180	53.3	60.2 (32-82)	80.6	4.4	3.0	22	6.6	4
	Gem + Irinotecan	180	57.2	63.2 (38-81)	82.2	16.1	3.5	21	6.3	
Moore *et al*[38]	Gem	284	57	64 (36.1-92)	75.0	8.0	3.55	17	5.91	4
	Gem + Erlotinib	285	47.7	63.7 (37-84)	76.5	8.6	3.75	23	6.24	
Cutsem *et al*[39]	Gem	347	58	62 (30-88)	77	8	3.6	24	6.06	4
	Gem + Tipifarnib	341	57	61 (29-89)	76	6	3.7	27	6.43	
Eckhardt *et al*[40]	Gem	120	59	60 (35-86)	73	NR	3.03	NR	7.36	5
	Gem + Tipifarnib	124	64	63 (35-81)	71	NR	2.3	NR	6.73	
Philip *et al*[37]	Gem	371	54	64.3	78	7	3.0	NR	5.9	4
	Gem + cetuximab	372	51	63.7	79	8	3.4	NR	6.3	
Kindler *et al*[36]	Gem	300	51	65.0 (35-86)	85	10	2.9	NR	5.9	4
	Gem + Bevacizumab	302	58	63.7 (26-88)	84	13	3.8	NR	5.8	
Kindler *et al*[35]	Gem	316	59	62 (35-89)	72	1.6	4.4	NR	8.3	5
	Gem + Axitinib	316	61	61 (34-84)	72	4.9	4.4	NR	8.5	
Spano *et al*[41]	Gem	34	47	61.0 (36-78)	19	3	3.7	23.5	5.6	4
	Gem + Axitinib	69	51	65.0 (44-81)	40	7	4.2	36.8	6.9	
Friess *et al*[34]	Gem	43	42	66 (56-80)	91	14	3.83	0.24	7.7	4
	Gem + Cilengitide	46	57	68 (40-80)	94	17	3.66	0.15	6.7	
Richards *et al*[33]	Gem	44	72.7	64.1 (41-83)	86.4	5.3	3.0	17	5.1	4
	Gem + Enzastarin	86	53.5	68.3 (39-86)	90.7	8.6	3.4	19	5.6	
Bramhall *et al*[31]	Gem	119	71	62 (37-85)	62	16	3.2	17	5.46	5
	Gem + Marimastat	120	69	62 (32-83)	59	11	3.08	18	5.51	
Richards *et al*[32]	Gem	85	60.2	65 (36-83)	83.0	13.9	3.43	NR	7.13	5
	Gem + CA-994	85	59.3	62 (32-82)	82.6	11.8	3.06	NR	6.47	
Oettle *et al*[30]	Gem	282	53.5	63 (28-82)	91.1	7.1	3.3	20.1	6.3	4
	Gem + PPemetrexed	283	60.4	63 (27-82)	90.1	14.8	3.9	21.4	6.2	

NR: No record; PFS: Progression-free survival; OS: Overall survival.

(number of partial and complete responses, as defined by Response Evaluation Criteria in Solid Tumors[10]), PFS (time from randomization to progression or death) and OS (time from the date of random assignment until date of death or date last known to be alive) were based on the intent-to-treat population, consisting of all patients randomly assigned onto every study. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, and based on the safety population. The median PFS and median OS were assessed using the paired *t*-test. $P < 0.05$ was considered statistically significant.

Meta-analysis was performed using the Review Manager (version 5.1, provided by The Cochrane Collaboration). The strength of the associations between treatment and outcomes were estimated by risk ratio (RR, a ratio

Table 2 Grade 3 or 4 toxicity of selected clinical trials

Ref.	Intervention	Vomiting	Diarrhea	Neutropenia	Anemia	Thrombocytopenia
Louvet *et al*[15]	Gem	3.2	1.3	27.6	10.3	3.2
	Gem + Oxaliplatin	8.9	5.7	20.4	6.4	14.0
Poplin *et al*[16]	Gem	7	4	33	10	13
	Gem + Oxaliplatin	16	6	22	6	11
Heinemann *et al*[17]	Gem	5.9	4.7	NR	10.6	10.6
	Gem + Cisplatin	22.2	3.3	NR	13.3	4.4
Colucci *et al*[18]	Gem	2	0	9	4	2
	Gem + Cisplatin	2	4	18	6	2
Colucci *et al*[19]	Gem	< 1	1.5	13.7	1	18.9
	Gem + Cisplatin	2.6	< 1	24.7	4.8	15.3
Kulke *et al*[20]	Gem	24.1	NR	82.8	20	43.1
	Gem + Cisplatin	29	NR	74.2	25.8	79
	Gem + Irinotecan	16.7	NR	41.7	8.3	23.3
Berlin *et al*[21]	Gem	8	NR	5	10	11
	Gem + 5-fluorouracil	7	NR	7	10	19
Herrmann *et al*[22,24]	Gem	4	2	19	6	8
	Gem + Capeitabine	4	5	23	8	4
Cunningham *et al*[23]	Gem	6	4	22	6	6
	Gem + Capeitabine	6	5	35	4	11
Scheithauer *et al*[25]	Gem	0	0	7.6	0	2.5
	Gem + Capeitabine	0	5	10	5	0
Costanzo *et al*[26]	Gem	0	0	2	6	0
	Gem + 5-fluorouracil	2	0	2	7	2
Abou-Alfa *et al*[27]	Gem	3.1	< 1	14.6	7.6	4.4
	Gem + Exatecan	5.3	1.1	30.3	5.9	15.4
Stathopoulos *et al*[28]	Gem	1.4	2.8	15.7	4.2	0
	Gem + Irinotecan	1.6	3.2	26.6	5	5
Lima *et al*[29]	Gem	8.2	1.8	31.9	13	14.2
	Gem + Irinotecan	13.9	18.5	37.6	16.2	19.6
Moore *et al*[38]	Gem	NR	< 1	27%	NR	11%
	Gem + Erlotinib	NR	2	24%	NR	10%
Cutsem *et al*[39]	Gem	9	3	30	16	12
	Gem + Tipifarnib	7	4	40	20	15
Eckhardt *et al*[40]	Gem	9.3	0	33.9	11	15.3
	Gem + Tipifarnib	4	0	35.5	12.1	16.1
Philip *et al*[37]	Gem	2.2	2.5	23.9	6.2	8.5
	Gem + cetuximab	6.6	2.8	23.3	9.7	6.6
Kindler *et al*[36]	Gem	NR	NR	29	8	12
	Gem + Bevacizumab	NR	NR	33	5	12
Kindler *et al*[35]	Gem	3.2	1.6	< 1	< 1	< 1
	Gem + Axitinib	3.9	1.3	0	0	0
Spano *et al*[41]	Gem	10	0	33	17	13
	Gem + Axitinib	6	6	28	8	20
Richards *et al*[33]	Gem	0	2.6	28.2	5.1	25.6
	Gem + Enzastarin	2.4	3.7	18.3	3.7	14.6
Bramhall *et al*[31]	Gem	14%	NR	NR	7%	NR
	Gem + Marimastat	6%	NR	NR	3%	NR
Richards *et al*[32]	Gem	9	3	NR	5	11
	Gem + CA-994	9	5	NR	13	25
Oettle *et al*[30]	Gem	3.7	0.7	12.8	2.9	6.2
	Gem + Pemetrexed	3.3	2.9	45.1	13.9	17.9

NR: No record.

of the probability of the event occurring in the exposed group versus a non-exposed group) and 95% confidence interval (CI)[11]. The heterogeneities between different studies or different subgroups were estimated using Cochran's Q test[12,13]. In addition, the I^2 value was used for evaluating the extent of heterogeneity between studies or subgroups[14]. The I^2 index measures the extent of true heterogeneity dividing the difference between the result of the Q test and its degrees of freedom by the Q value itself, and multiplied by 100. I^2 indicated the possibility of the variability among effect sizes caused by true heterogeneity between studies, not by sampling error[14]. If a significant Q test ($P < 0.1$) or $I^2 > 50\%$ indicated that heterogeneity existed between studies, the random effects model was used for meta-analysis, otherwise the fixed effects model was used. Publication bias was assessed by visual inspection of funnel plots.

RESULTS

Literature search and trial flow

The results of the literature search are depicted in Figure

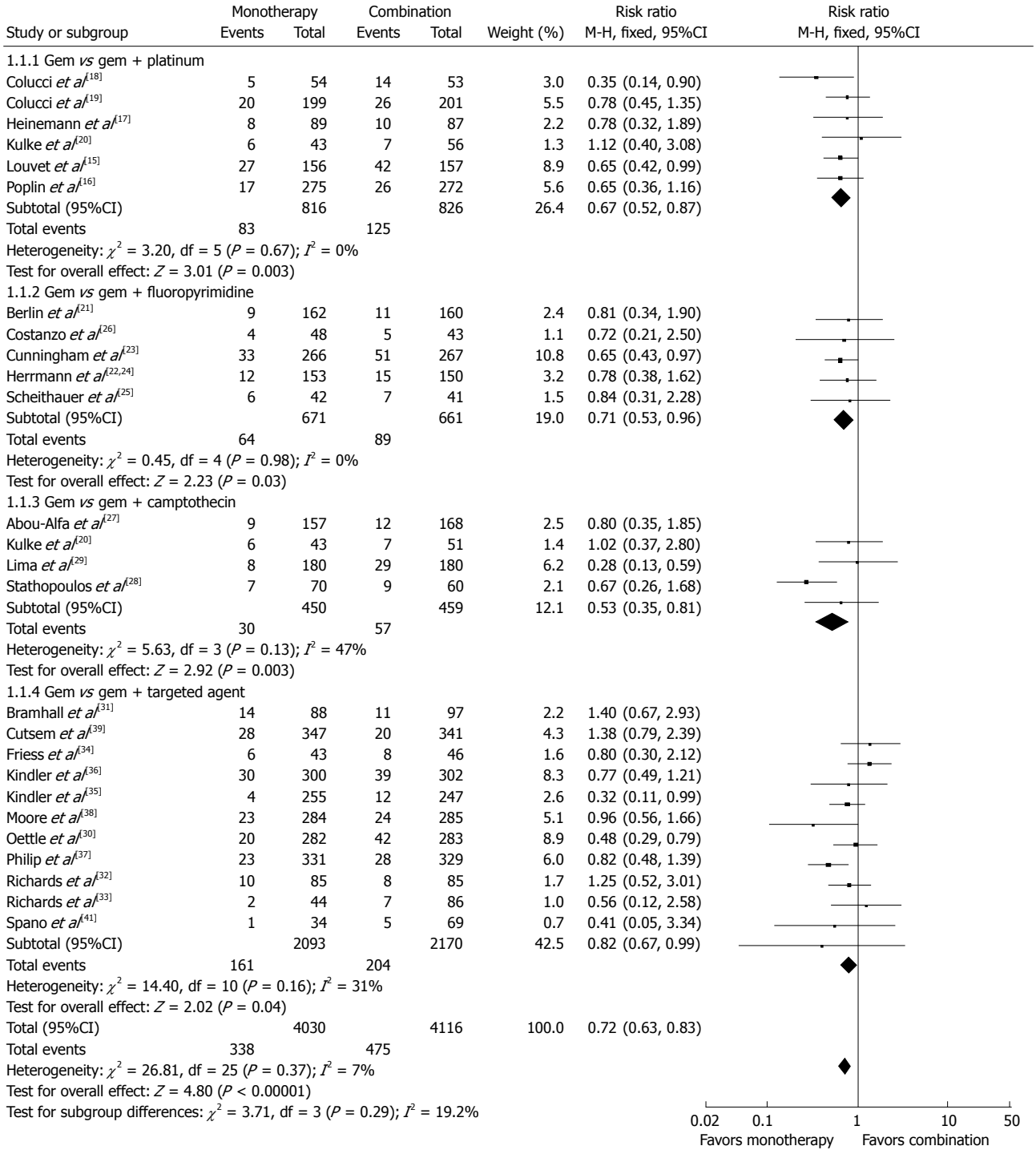


Figure 2 Fixed effect model of risk ratio of objective response rate. CI: Confidence interval.

1. Some 2707 studies were initially retrieved. After reading the title and abstract, 2675 studies were excluded, and 32 studies were left for further review. Six of these were excluded because of poor quality or failure to meet the inclusion criteria.

Characteristics of selected trials

The characteristics of the included studies are shown in Table 1. Twenty-six studies were included in the present analysis, with 8808 recruited patients. The studies were divided into four subgroups based on types of cytotoxic agents used, including platinum[15-20], fluoropyrimidine[21-26], camptothecin[21,27-29] and targeted agents[30-41]. All included studies enrolled patients that were ⩾ 18 years; had a histologically confirmed locally advanced or metastatic pancreatic cancer not amenable to surgical resection; no previous chemotherapy; a life expectancy of at least 12 wk; a Karnofsky performance status ⩾ 50, or World Health Organization performance status of 0-2; and adequate liver, renal, and hematopoietic functions.

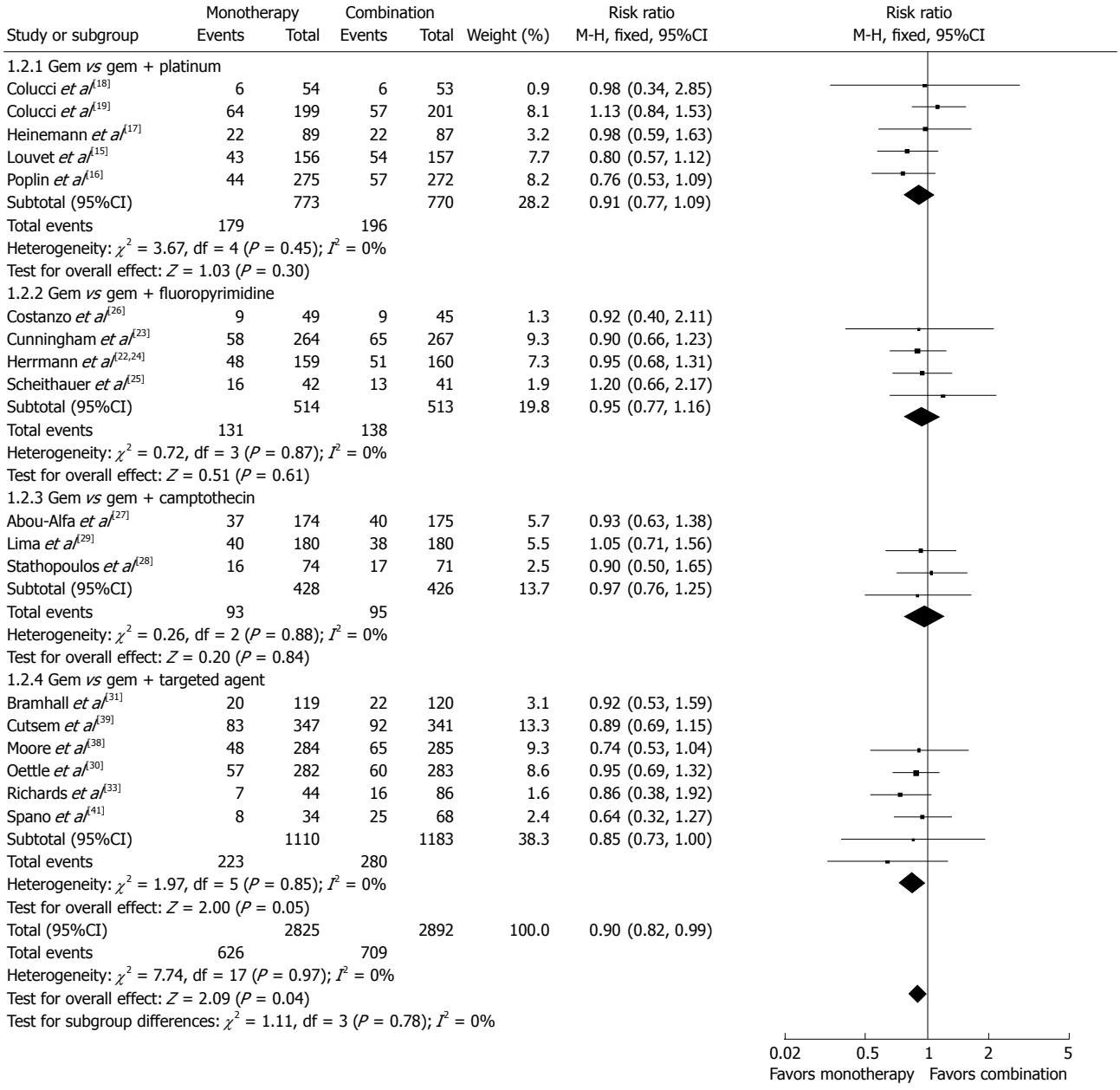


Figure 3 Fixed effect model of risk ratio of 1-year overall survival rate. CI: Confidence interval.

The details of toxicity assessment are shown in Table 2 (only grade 3 and 4 toxic effects are presented), including vomiting, diarrhea, neutropenia, anemia and thrombocytopenia.

The methodological quality of selected studies was high. Most trials had a Jadad score of 3, while three studies had a score of 4. Methods of double blinding were infrequently reported.

Gemcitabine combination therapy improves objective response rate compared with gemcitabine treatment alone

This analysis evaluated 26 trials (8146 patients) comparing gemcitabine monotherapy with the combination of gemcitabine and some other cytotoxic agent. Based on all studies, patients treated with gemcitabine monotherapy had a significantly lower ORR than gemcitabine combination therapy (RR, 0.72; 95%CI: 0.63-0.83; $P < 0.001$). The RRs in the different subgroups were 0.67, 0.71, 0.53 and 0.82, for platinum, fluoropyrimidine, camptothecin and targeted agents, respectively. All RRs in the subgroups were also significant. Data are shown in Figure 2.

Gemcitabine combination therapy improves 1-year overall survival rate compared with gemcitabine alone

5717 patients from 18 trials were included in this meta-analysis comparing gemcitabine monotherapy with combination therapies for 1-year overall survival. The RRs of 1-year overall survival were analyzed both totally and in the different subgroups. Subgroup analysis showed that the RRs of the monotherapy-based 1-year overall survival were lower than for the combination groups (RR:

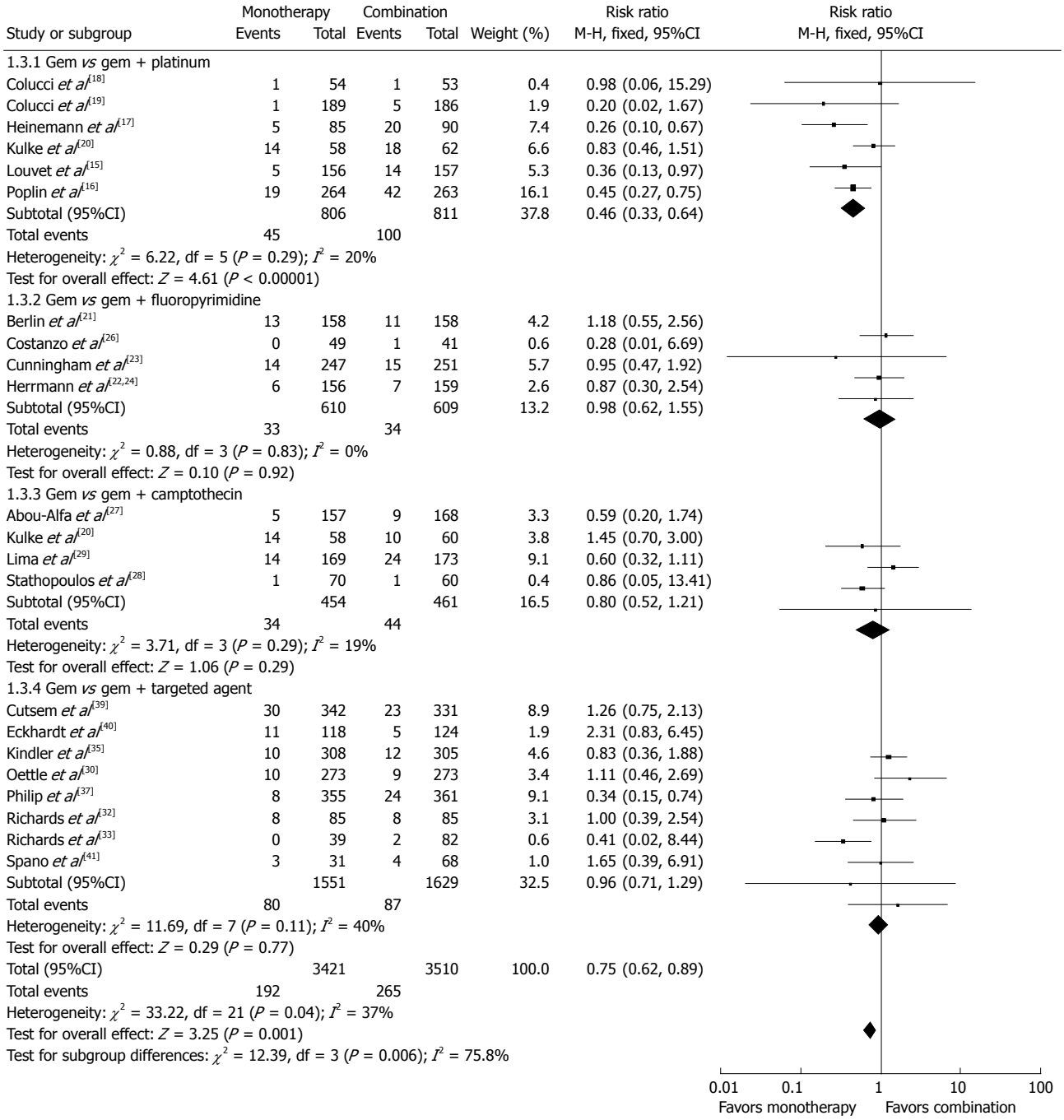


Figure 4 Fixed effect model of risk ratio of grade 3 or 4 vomiting. CI: Confidence interval.

0.91, 0.95, 0.97 and 0.85, for platinum, fluoropyrimidine, camptothecin and targeted agents, respectively), but no significant differences were found. When analyzed in terms of total events, the RR shown in Figure 3 was 0.90 [(95%CI: 0.82-0.99); P = 0.04]. The result showed that the 1-year overall survival rate of gemcitabine monotherapy was almost 90% of that of the combination therapy.

Gemcitabine combination therapy increases the toxicity effect compared with gemcitabine alone

Outcomes of the meta-analysis of the main toxicities are presented in Figures 4-8. All RRs with grade 3-4 toxicities analyzed in this study were lower in the gemcitabine monotherapy group than in the combination group. The incidence of vomiting (RR, 0.75; 95%CI: 0.62-0.89; P = 0.001), diarrhea (RR, 0.66; 95%CI: 0.49-0.89; P = 0.006) and thrombocytopenia (RR, 0.76; 95%CI: 0.60-0.97; P = 0.03) were all significantly different between the treatment groups, while no significant difference was noted regarding neutropenia (RR, 0.88; 95%CI: 0.72-1.06; P = 0.18) and anemia (RR, 0.96; 95%CI: 0.82-1.12; P = 0.60).

In subgroup analysis, the RRs were 0.46-0.98 for vomiting, 0.65-0.70 for diarrhea, 0.70-1.03 for neutropenia, 0.87-1.09 for anemia and 0.65-0.85 for thrombocy-

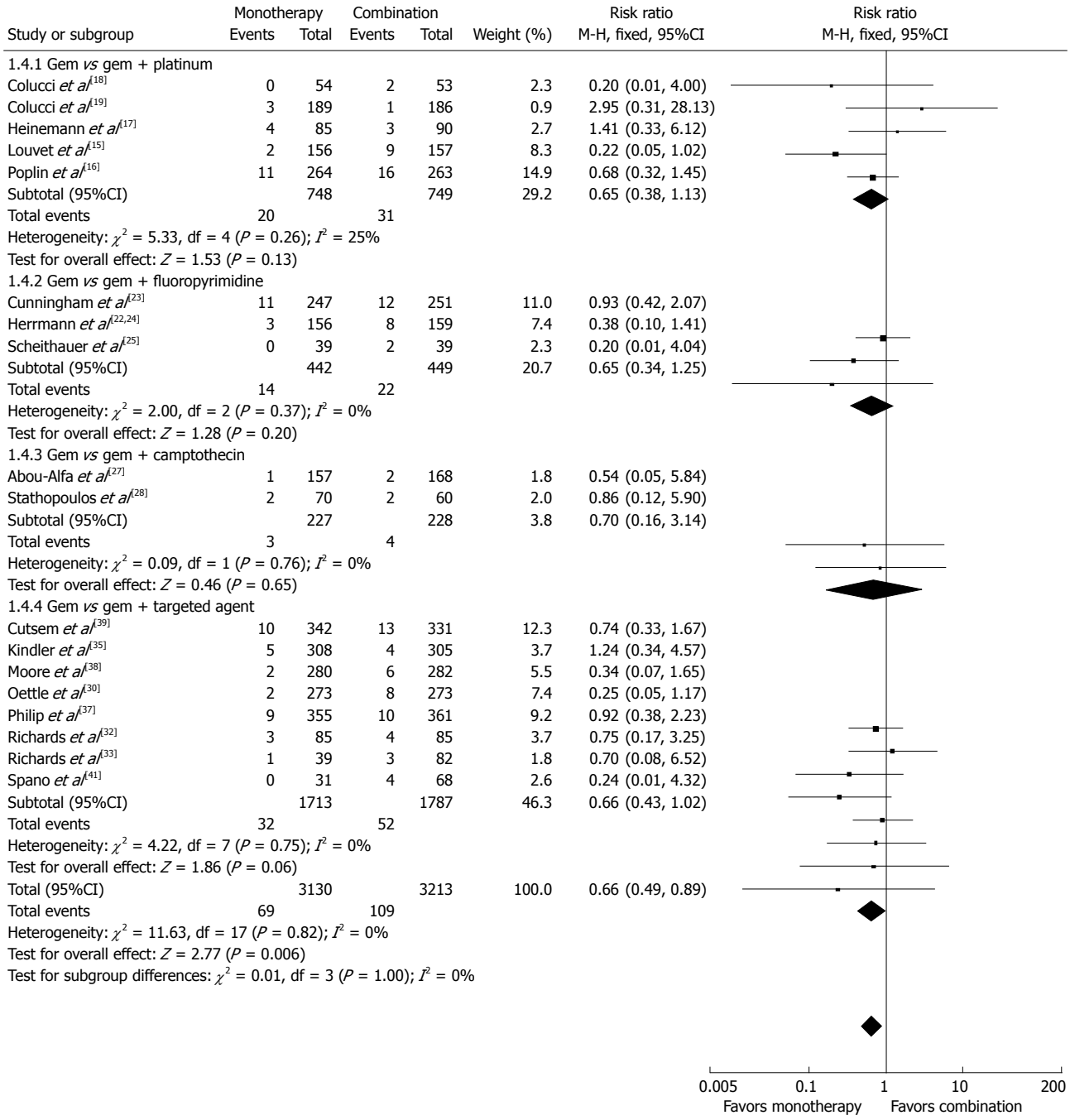


Figure 5 Fixed effect model of risk ratio of grade 3 or 4 diarrhea. CI: Confidence interval.

topenia, respectively. No subgroup differences in overall toxic outcomes, expect for vomiting, were found between the gemcitabine monotherapy group and the combination groups. Gemcitabine monotherapy decreased the incidence rate of vomiting by more than 50% compared with the gemcitabine plus platinum subgroup (RR, 0.46; 95%CI: 0.33-0.64; $P < 0.001$), while no significant differences for other subgroups was seen. In addition, the results showed that gemcitabine plus fluoropyrimidine therapy significantly increased the incidence of neutropenia (RR, 0.70; 95%CI: 0.55-0.88; $P = 0.002$).

The median PFS and median OS between gemcitabine monotherapy and combination therapies

The data of median PFS and OS in every study were extracted and assessed by a paired *t*-test. The results showed that only gemcitabine plus fluoropyrimidine significantly increased the median PFS (3.480 *vs* 4.520; $P = 0.045$) and median OS (6.950 *vs* 7.840; $P = 0.038$). Data are shown in Figure 9.

Publication bias

The funnel plots are shown in Figure 10. There was no

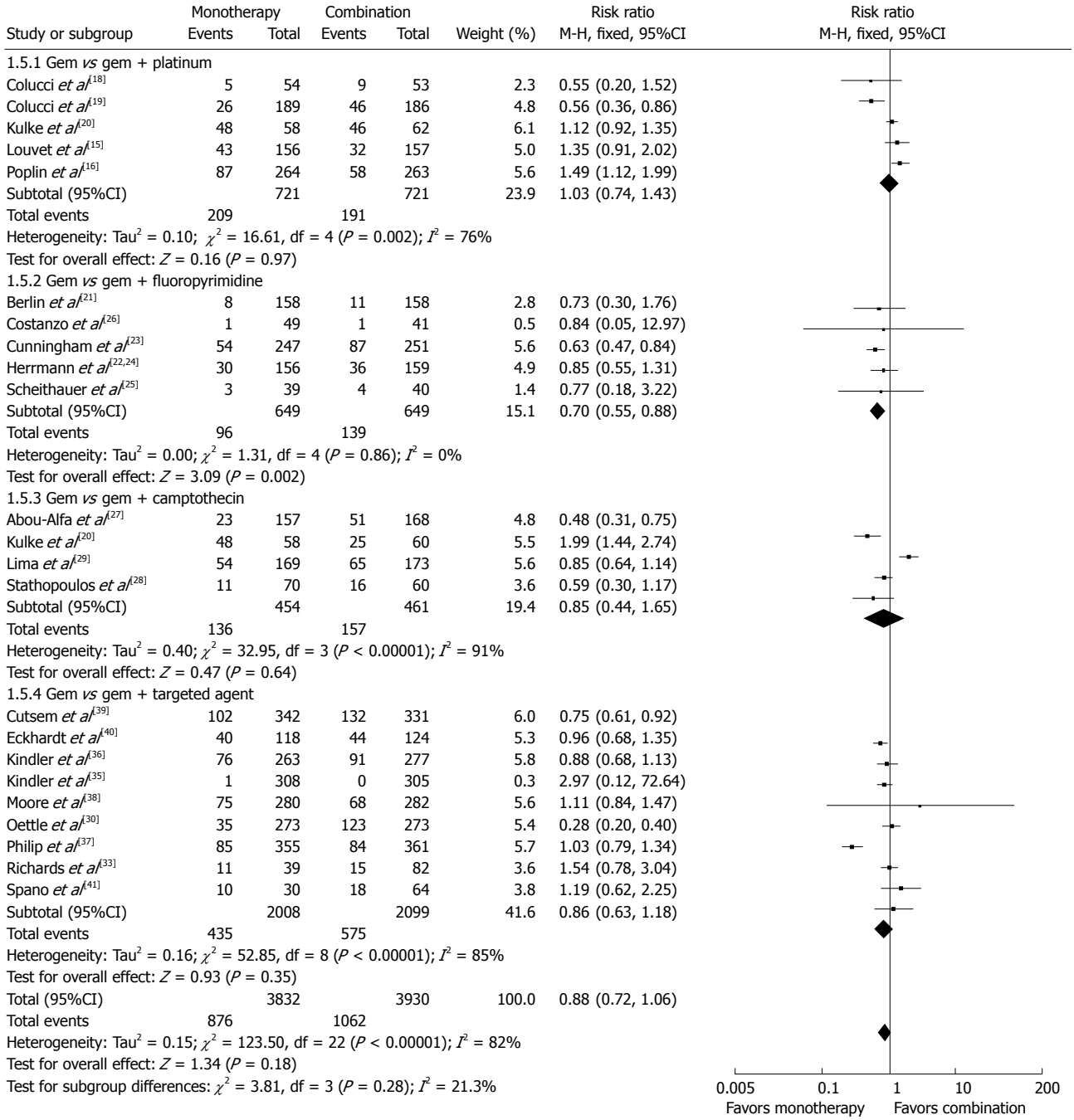


Figure 6 Random effect model of risk ratio of grade 3 or 4 neutropenia. CI: Confidence interval.

obvious publication bias found in the analysis.

DISCUSSION

Pancreatic cancer is a devastating disease with a dismal prognosis. Most of patients present with locally advanced or metastatic disease and are thus not candidates for surgical resection, thereby having to rely on palliative chemotherapy as the only treatment option. In this situation, the goals of chemotherapy should be to control tumor progression, decrease toxicity, and improve the survival rate. At present, gemcitabine monotherapy remains the standard treatment for patients with locally advanced or metastatic pancreatic cancer, but its efficacy is unsatisfactory. During the last decade, several randomized controlled clinical trials have evaluated gemcitabine in combination with various agents in an attempt to improve the prognosis of pancreatic cancer. The aim of this meta-analysis was to compare the therapeutic efficacy of gemcitabine-based combination treatments with gemcitabine alone in patients with locally advanced or metastatic pancreatic cancer. The results of this study showed that various combination therapies overall did not provide any major benefit compared with gemcitabine monotherapy.

Moreover, gemcitabine monotherapy had an almost 30% lower ORR than combination therapy regimens, but

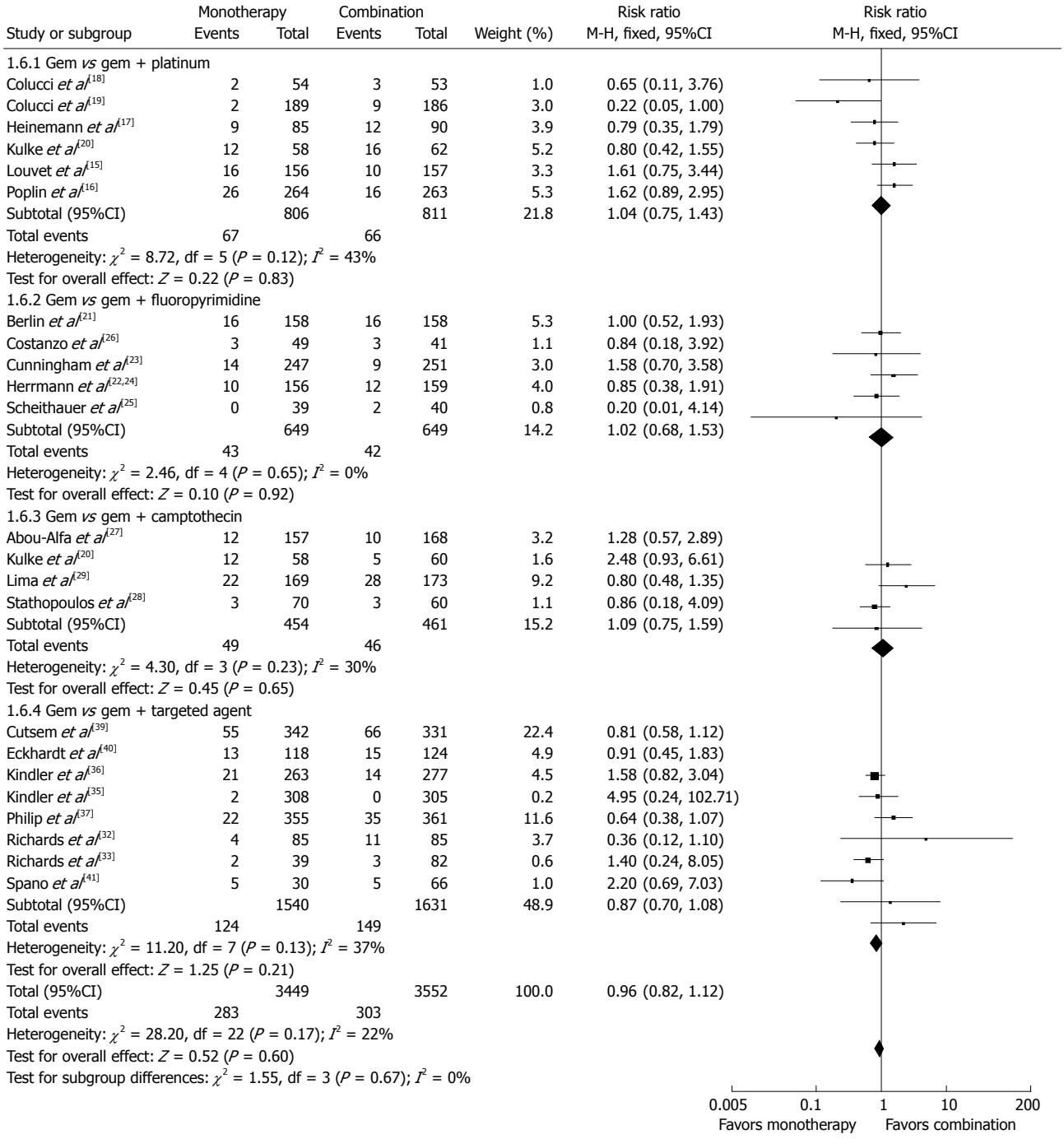


Figure 7 Fixed effect model of risk ratio of grade 3 or 4 anemia. CI: Confidence interval.

only had a 10% lower 1-year OS than combination therapy. According to the present data, 1-year OS in advanced or metastatic pancreatic cancer was low (less than 20%) after gemcitabine monotherapy; therefore, the combination therapy options studied did not improve outcome in a substantial way, the prognosis still being poor[21,31,42]. In addition, there were no significant improvements found in this analysis in median PFS and median OS after combination therapy compared with monotherapy, except for gemcitabine plus fluoropyrimidine treatment. Our results are similar to previously published analyses[23,43,44]. We also assessed the five most common toxicities related to chemotherapeutic treatment of locally advanced or metastatic pancreatic cancer, including hematological and gastrointestinal side effects. Grade 3-4 toxicities tended to be higher following combination therapy compared with monotherapy, and three of the five investigated toxicities reached significant differences. The RRs of vomiting, diarrhea, and thrombocytopenia in the monotherapy group were about 70% of that noted in the combination therapy group. Furthermore, subgroup analysis showed that the RR of vomiting after treatment with gemcitabine plus platinum was twice that of monotherapy. The results presented here showed that combination therapy induced

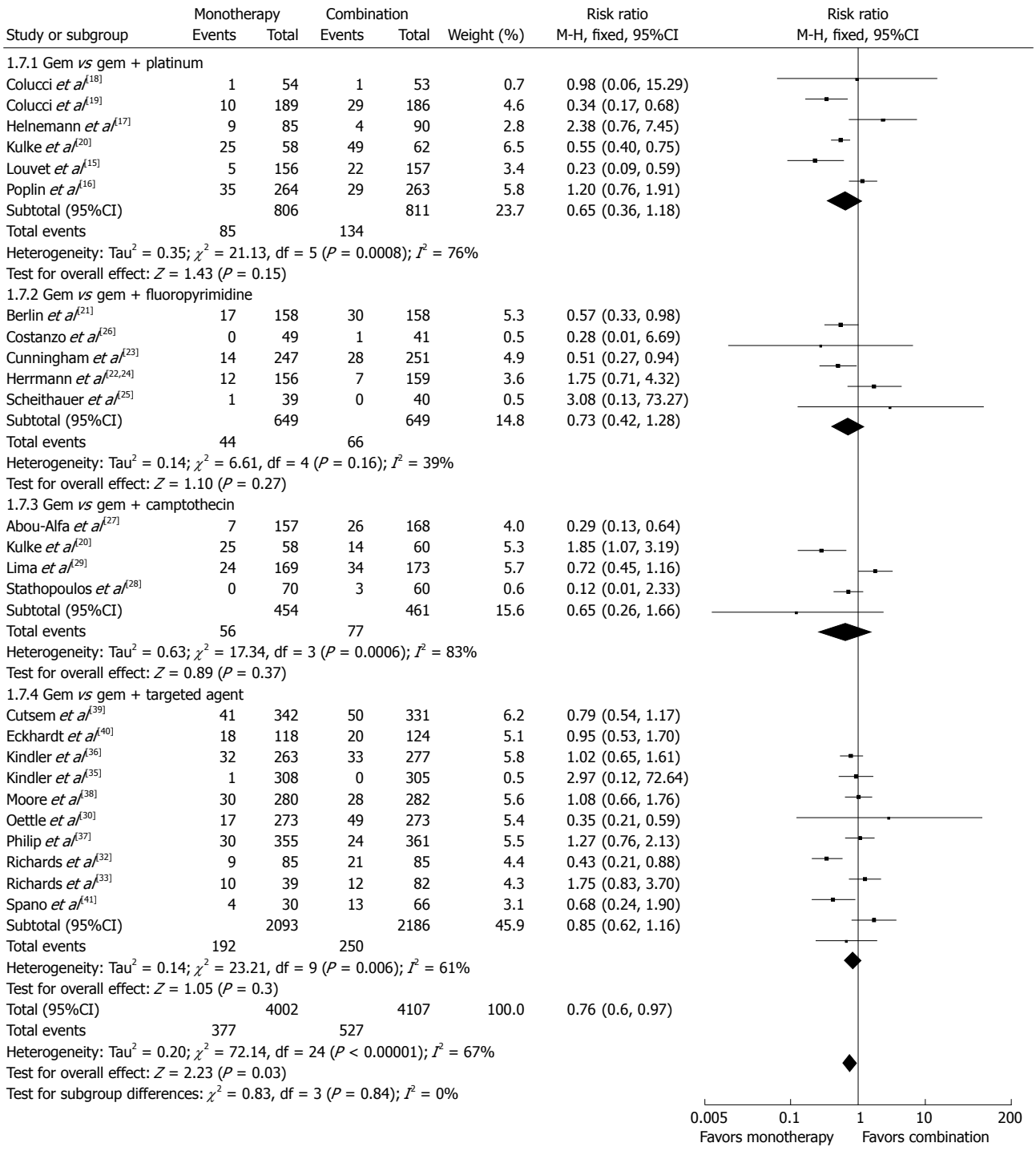


Figure 8 Random effect model of risk ratio of grade 3 or 4 thrombocytopenia. CI: Confidence interval.

more toxicity than gemcitabine alone. Similar results were found in a previous study[45]. This finding might explain why combination therapy received higher ORR, but without affecting the overall prognosis, as toxicities counterbalanced the positive effects.

In the present analysis, we included several kinds of combination therapies with gemcitabine, which could provide different anti-tumor effects and different uptake and toxicity profiles, including platinum based agents, topoisomerase inhibitors, taxanes, bevacizumab, cetuximab and other biologically targeted agents. However, no significant differences were found between the subgroups, except for side-effects (vomiting). In addition, the analysis of heterogeneity was not significant in most subgroups. This is interesting, and indicates that effectiveness of the different combinations regarding outcomes could not clearly be shown. There are two possible reasons that could explain these results. One is related to gemcitabine-uptake receptors. The other one could be the inherent drug-resistant character of pancreatic carcinoma cells and

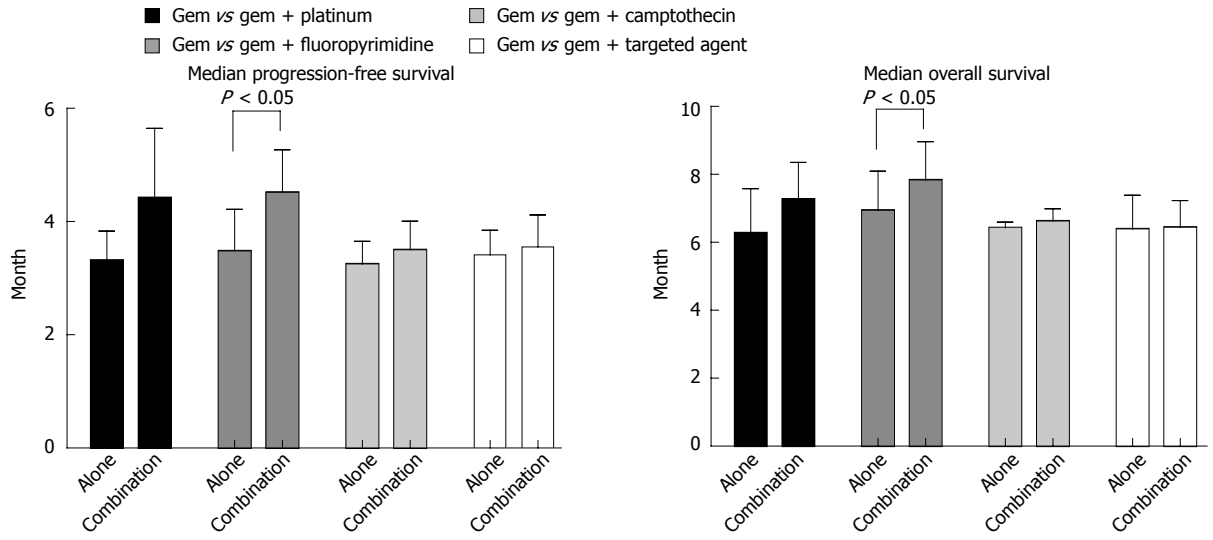


Figure 9 Median progression-free survival and overall survival between gem-monotherapy and combination therapy.

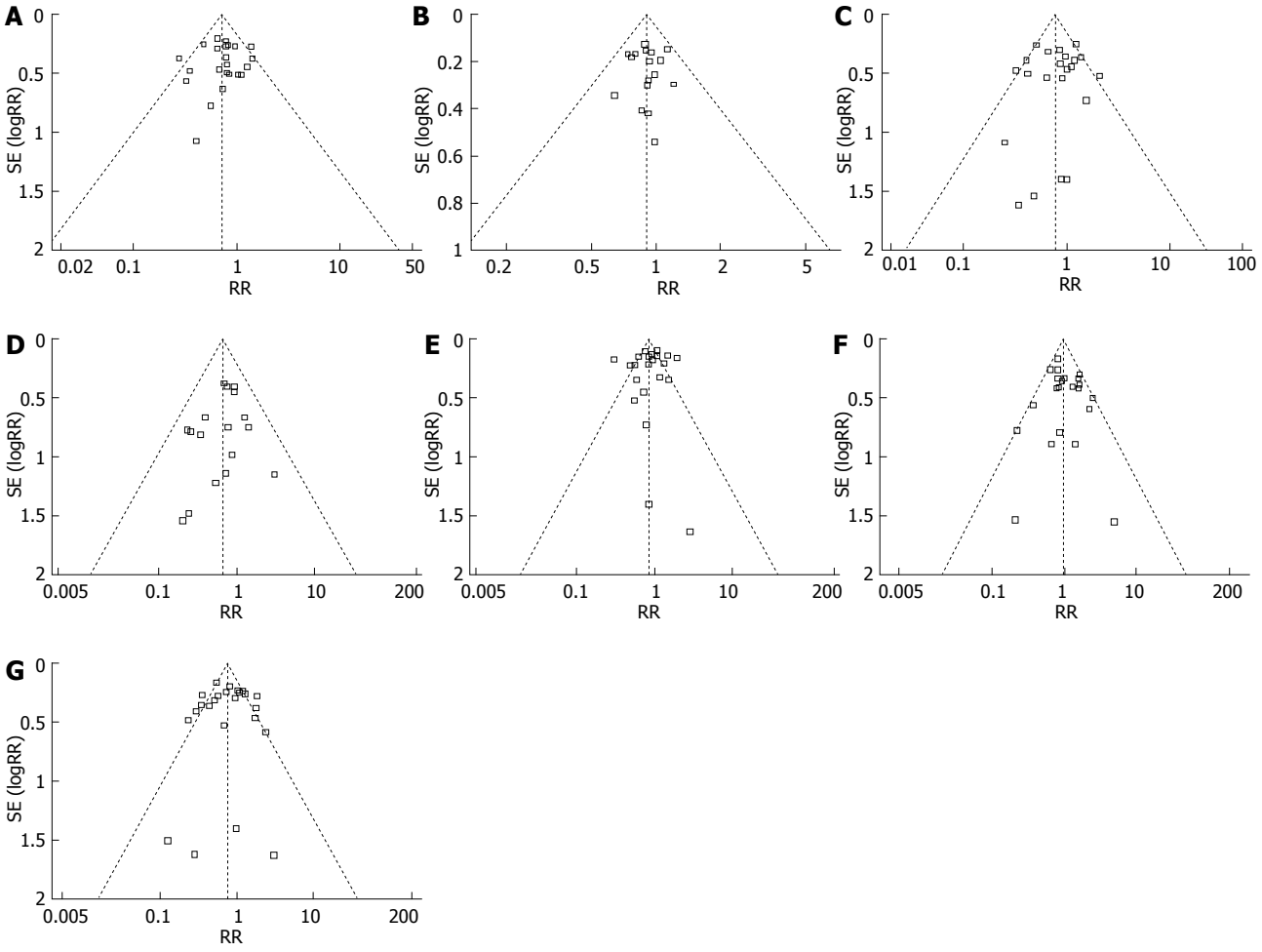


Figure 10 Funnel plots. A: Objective response rate; B: 1-year overall survival rate; C: Vomiting; D: Diarrhea; E: Neutropenia; F: Anemia; G: Thrombocytopenia. RR: Risk ratio.

the effect of the surrounding stroma.

Human equilibrative nucleoside transporter-1 (hENT-1) is the main membrane channel responsible for intracellular bioavailability of gemcitabine. Preclinical and clinical data have revealed that only a minority of patients have high hENT1 expression and reduced levels of hENT-1 expression correlate with increased resistance to gemcitabine[7]. To increase gemcitabine efficiency and

avoid overtreatment, determination of the hENT1 status at the time of diagnosis and modifying gemcitabine to allow it to bypass the receptor may represent a future mode of overcoming gemcitabine resistance[7]. Multidrug resistance proteins, including ABC transporters, have also been implied in drug resistance in pancreatic cancer and limit the efficacy of gemcitabine[46]. The pancreatic stellate cell has a key role in stroma formation and secretes factors that promote pancreatic tumor growth and resistance to radiation and gemcitabine chemotherapy[47]. The hypoxic stroma could be a physical barrier preventing chemotherapeutic drugs from reaching the pancreatic cancer cells per se, and depletion of the stroma could enhance cancer drug delivery[48]. Recently, cancer stem cells and epithelial to mesenchymal transition (EMT)-type cells have been implicated in metastasis formation and drug resistance[49]. Selective targeting of cancer stem cells and EMT-type cells might represent a future strategy to enhance chemosensitivity. The plasma circulating time of gemcitabine is short and its rapid metabolism thus limits tumor uptake. Using novel formulas such as PEGylated gemcitabine may result in a prolonged circulation time, and possibly inducing cytotoxicity at lower concentrations than those required with treatment with the native drug[50]. Pancreatic cancer, including its concurrent stroma, is complex; therefore, treatment with a single anti-tumor agent may not be effective enough. Determining the underlying mechanisms of drug resistance in pancreatic cancer will provide ways of overcoming chemoresistance and provide a foundation for novel therapies.

During the last decade, several meta-analyses have focused on the efficacy of combination therapy in locally advanced or metastatic pancreatic cancer[43,44,51-54]. Most of these studies have reported positive trends or have concluded that combination treatments may improve outcome, but only a limited number of the studies have reported toxicity data, or have only focused on one anti-tumor agent. In the present study, we collected and analyzed the outcomes in different chemotherapeutic subgroups, as well as toxicity, to make the result more comprehensive and convincing. However, several limitations in this analysis should still be considered. Firstly, some endpoints were not evaluated in all of the included randomised controlled trials. Secondly, heterogeneity of certain endpoints in some subgroups might limit the comparability between studies and affect the validity, despite the use of randomized models.

In conclusion, we compared the outcomes of different combination therapies with gemcitabine monotherapy, including ORR, OS, PFS and major toxicity. The results showed that gemcitabine combination therapy leads to a modest improvement in survival, though with more toxicity reported compared with gemcitabine monotherapy.

COMMENTS

Background

Pancreatic cancer is a devastating disease with a dismal prognosis. The majority of patients (80%-90%) have unresectable disease at diagnosis and have to rely on palliative chemotherapy alone. In this situation, the goals of chemotherapy should be to control tumor progression and improve the survival rate with minimal toxicity.

Research frontiers

Gemcitabine has long been the first-line chemotherapeutic agent in pancreatic cancer. However, because of high levels of intrinsic and acquired chemoresistance, a large number of patients do not respond to the drug. In an attempt to improve clinical efficacy, systemically administered gemcitabine has been combined with a second cytotoxic agent, such as platinum analogs, fluoropyrimidine, or a targeted cytotoxic agent. However, the added value of these additive agents is unknown.

Innovations and breakthroughs

The present meta-analysis included 26 high-quality randomized trials comparing gemcitabine monotherapy with gemcitabine combination therapy. The primary outcome variables evaluated were objective response rate, overall survival, progression-free survival and major toxicity. The results showed that gemcitabine combination therapy was associated with only a modest improvement in survival, though with more toxicity reported, compared with gemcitabine monotherapy.

Applications

The results of this study imply that current treatments for unresectable pancreatic cancer are not sufficiently effective. Further research concerning the mechanisms involved in drug resistance in pancreatic cancer could discover ways of overcoming chemoresistance and lead to novel types of therapy.

Peer review

This is a meta-analysis concerning combination chemotherapy with Gemcitabine unresectable pancreatic cancer. The manuscript is well written and represents elaborative work.

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CASE REPORT

Cameron ulcers: An atypical source for a massive upper gastrointestinal bleed

Samir Kapadia, Sophia Jagroop, Atul Kumar

Samir Kapadia, Department of Medicine, Stony Book University School of Medicine, Stony Brook, NY 11794, United States
Sophia Jagroop, Atul Kumar, Gastroenterology and Hepatology, Northport VA Medical Center, Northport, NY 11768, United States
Author contributions: Kapadia S, Jagroop S, and Kumar A contributed equally to this work; Jagroop S and Kumar A performed the procedures; Kapadia S and Jagroop S wrote the paper.
Correspondence to: Atul Kumar, MD, Assistant Professor of Medicine, Gastroenterology and Hepatology, Northport VA Medical Center, 79 Middleville Rd., Northport, NY 11768, United States. atul.kumar2@va.gov
Telephone: +1-631-88978511 Fax: +1-631-4866113
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Abstract

Cameron lesions represent linear gastric erosions and ulcers on the crests of mucosal folds in the distal neck of a hiatal hernia (HH). Such lesions may be found in upto 50% of endoscopies performed for another indication. Though typically asymptomatic, these may rarely present as acute, severe upper gastrointestinal bleed (GIB). The aim is to report a case of a non-anemic 87-year-old female with history of HH and atrial fibrillation who presented with hematemesis and melena resulting in hypovolemic shock. Repeat esophagogastroduodenoscopy was required to identify multiple Cameron ulcers as the source. Endoscopy in a patient with HH should involve meticulous visualization of hernia neck and surrounding mucosa. Cameron ulcers should be considered in all patients with severe, acute GIB and especially in those with known HH with or without chronic anemia.

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Key words: Cameron lesion; Upper gastrointestinal bleeding; Shock; Hiatal hernia

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INTRODUCTION

The incidence of hiatal hernia (HH) rises with age[1]. Given the rising demographics and the growing number of endoscopies, this condition now constitutes an increasingly common endoscopic finding. One study reported the incidence to be upwards of 50% during upper endoscopies performed for another indication[2]. Though typically asymptomatic, several complications can occur including gastroesophageal reflux disease[3], iron deficiency anemia[4], acute or chronic bleeding[5], and ulcer or erosion formation[2]. Usually an incidental endoscopic finding, Cameron lesions represent linear gastric erosions and ulcers on the crests of mucosal folds in the distal neck of a HH. Both erosions and ulcers are thought to be distinct forms of the same disease process. Lesions are found in 5.2% of patients with HH identified on upper endoscopy[6] and in over 60%, multiple lesions may be found[1].

CASE REPORT

An 87-year-old female presented to the Emergency Department (ED) with several episodes of bright red hematemesis and black, tarry stools over six hours. She complained of severe lightheadedness and crampy, lower abdominal pain. On examination, she was found to be hypotensive with systolic blood pressures in the 60s, and despite aggressive resuscitation with intravenous fluids

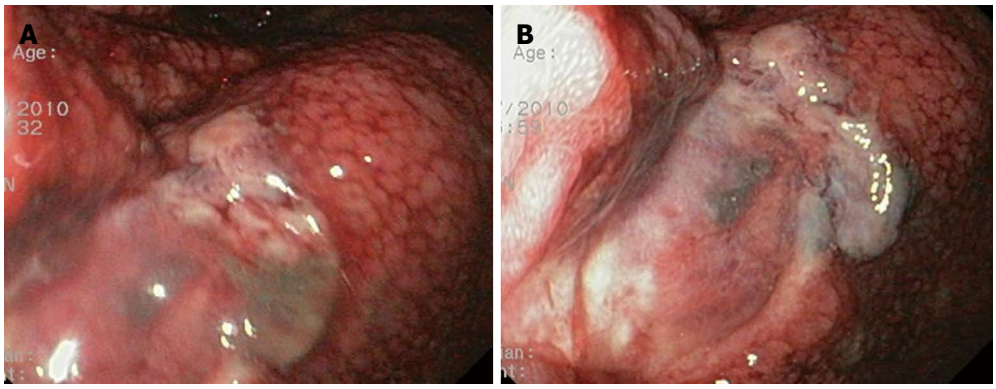


Figure 1 Ulcer with vessel in hernia sac. A: Retroflexed view of cameron ulcer; B: Second ulcer.

and blood products, she required vasopressors.

Two days earlier, she had been discharged following a brief hospital stay for atrial fibrillation and a large HH. She had been conservatively treated with proton pump inhibitor, Sucralfate, and Ondansetron as needed with fair results.

At this presentation, hemoglobin (Hgb) was 12.7 g/dL (baseline of 14.8 g/dL). Nasogastric lavage returned 500 cc of bright red blood. Intravenous proton pump inhibitor was started, patient was intubated and an emergent EGD revealed a large clot in the gastric fundus along with diffuse, friable mucosa in the mid-distal esophagus. No other bleeding site was identified. Despite multiple units of packed red blood cells and fresh frozen plasma, the patient's hemoglobin continued to decline. A repeat EGD demonstrated multiple circumferential non-bleeding Cameron ulcers at 37 cm with large placental clots in the fundus (Figure 1). Small clots were also seen at the gastroesophageal junction. In total, the patient received 14 units of packed red blood cells, multiple units of fresh frozen plasma, protamine sulfate and Vitamin K. Her Hgb stabilized on hospital day 10. During the course, she developed aspiration pneumonia, which was successfully treated with antibiotics. No further bleeding occurred and the patient recovered.

DISCUSSION

The pathogenesis of Cameron lesions is poorly understood. Some attribute lesion formation to mechanical trauma to the esophagus caused by respiration-related diaphragmatic contractions[6]. Other etiological factors may include acid reflux, ischemia, *Helicobacter pylori* infection, gastric stasis or vascular stasis[7]. It is likely that the etiology is multifactorial, and includes genotype, phenotype and patient risk factors including underlying co-morbidities and medication use. The prevalence is also likely dependent on the size of the HH with a 10%-20% risk for Cameron ulcers in hernias 5 cm in size or greater[8]. However, the absence of HH should not rule out Cameron lesions either. One study used push enteroscopy to evaluate ninety-five patients with obscure GIB previously investigated with standard endoscopy. Of the thirty-nine patients with an identifiable source, Cameron lesions were the second most commonly missed lesion (21%)[9]. Presumably, the lack of a large HH may have reduced the suspicion for Cameron lesions. Among the most concerning clinical manifestations of Cameron lesions are acute and chronic GIB. Chronic blood loss resulting in anemia has been well described. A large prospective, national, population-based study found that patients with HH had a significantly higher association of iron-deficiency anemia as compared to those with esophagitis[4]. Additionally, in a case-controlled study, Cameron showed that of 259 patients with radiographic evidence of HH, 18 were anemic compared to 1 in the control group ($P < 0.001$)[10]. Patients with Cameron lesions typically respond well to medical treatment consisting of iron supplementation with or without acid-suppression therapy. It is worth noting that our patient's MCV was 97 on admission without any supplementation.

More alarming are lesions that present as severe, acute GIB. Studies have reported rates of 29%-58%, raising the possibility of life-threatening hemorrhage[2,10]. To our knowledge, there are very few reports of life-threatening upper GIB secondary to a Cameron ulcer. One case report describes the successful treatment of a visible vessel within a Cameron ulcer with band ligation[11]. However, in these cases, surgical intervention is recommended, as endoscopic hemostasis can be technically difficult. Potential risks of endoscopy in acute, upper GIB include deep ulcers and perforation as the gastrointestinal wall around the gastroesophageal junction lacks fibrous tissue. Thus, surgery should be considered in those with either severe sliding hernias with Cameron lesions and in patients with lesion-related complications refractory to medical treatment[12]. Long-term recurrence rates are extremely low following surgery[13].

Complicating our case was the need to anticoagulate the patient for atrial fibrillation prior to endoscopy. More studies are needed to evaluate whether radiographic evidence of large HH should prompt an endoscopy prior to anticoagulation. In conclusion, endoscopy in a patient with radiographic evidence of large HH should involve meticulous antegrade and retrograde visualization with views of hernia neck and surrounding mucosa. Cameron

ulcers are a potential source of severe, acute GIB in patients with known HH with or without chronic anemia.

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CASE REPORT

Metastases of esophageal carcinoma to skeletal muscle: Single center experience

Jan Cincibuch, Miroslav Mysliveček, Bohuslav Melichar, Čestmír Neoral, Iva Metelková, Michaela Zezulová, Hana Procházková-Študentová, Patrik Flodr, Miloslava Zlevorová, René Aujeský, Karel Cwiertka

Jan Cincibuch, Bohuslav Melichar, Michaela Zezulová, Hana Procházková-Študentová, Miloslava Zlevorová, Karel Cwiertka, Department of Oncology, Palacký University Medical School and Teaching Hospital, 77520 Olomouc, Czech Republic
Miroslav Mysliveček, Iva Metelková, Department of Nuclear Medicine, Palacký University Medical School and Teaching Hospital, 77520 Olomouc, Czech Republic
Čestmír Neoral, René Aujeský, 1st Department of Surgery, Palacký University Medical School and Teaching Hospital, 77520 Olomouc, Czech Republic
Patrik Flodr, Department of Pathology, Palacký University Medical School and Teaching Hospital, 77520 Olomouc, Czech Republic
Author contributions: Cincibuch J and Melichar B were involved in the conception of the paper, data acquisition and analysis and coordinated the writing of the manuscript; Mysliveček M, Neoral Č, Metelková I, Zezulová M, Procházková-Študentová H, Flodr P, Zlevorová M, Aujeský R and Cwiertka K participated in the data acquisition and analysis, and contributed to the writing of the manuscript; all authors read and approved the final version of the manuscript.
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Correspondence to: Bohuslav Melichar, MD, PhD, Professor and Head, Department of Oncology, Palacký University Medical School and Teaching Hospital, I.P. Pavlova 6, 77520 Olomouc, Czech Republic. bohuslav.melichar@fnol.cz
Telephone: +420-588-444288 Fax: +420-588-442522
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Abstract

Metastases of esophageal carcinoma to the skeletal muscle are rare, but the incidence may be increasing because of better diagnosis resulting from widespread use of positron emission tomography/computed tomography (PET/CT). A cohort of 205 patients with esophageal carcinoma treated at our center who had PET/CT between 2006 and 2010 was retrospectively evaluated for the presence of skeletal muscle metastases. Four patients had skeletal muscle metastases of esophageal carcinoma, including two patients with squamous cell carcinoma. In another patient with squamous cell carcinoma of the esophagus and synchronous skeletal muscle metastases, muscle metastases were subsequently shown to be related to second primary pancreatic adenocarcinoma. In all cases, skeletal muscle metastases were the first manifestation of systemic disease. In three patients palliation was obtained with the combination of external beam radiation therapy, systemic chemotherapy or surgical resection. Skeletal muscle metastases are a rare complication of esophageal carcinoma.

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Key words: Drug therapy; Esophageal neoplasms; Muscle; Skeletal; Positron-emission tomography; Radiotherapy

Peer reviewers: Luigi Bonavina, Professor, Department of Surgery, University of Milano, Via Decembrio 19A, 20137 Milano, Italy; Zhiheng Pei, Associate Professor, Department of Pathology and Medicine, New York University School of Medicine, 6001W, 423 East 23rd street, New York, NY 10010, United States; Dr. Giuseppe S Sica, Department of Surgical Science, Tor Vergata University of Rome, Viale Oxford 81, 00133 Rome, Italy; Dr. Sundeep Singh Saluja, Department of Gastrointestinal Surgery, GB Pant Hospital and MAulana Azad Medical College, W/9 West Patel Nagar, New Delhi 110008, India

Cincibuch J, Mysliveček M, Melichar B, Neoral Č, Metelková I, Zezulová M, Procházková-Študentová H, Flodr P, Zlevorová M, Aujeský R, Cwiertka K. Metastases of esophageal carcinoma to skeletal muscle: Single center experience. *World J Gastroenterol* 2012; 18(35): 4962-4966 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i35/4962.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i35.4962

INTRODUCTION

The prognosis of esophageal carcinoma is, in general, poor, with the mortality rate reaching 90% of the incidence rate[1]. Both uncontrolled growth of the primary tumor or locoregional recurrence and distant metastases may be the ultimate cause of death in individual patient.

Esophageal carcinoma commonly spreads to the liver, lung, adrenals, bone, pleura, peritoneum or pericardium. Less frequent sites of metastases include thyroid, pancreas, spleen, ovary, kidney, heart, skin, gallbladder or small and large bowel[2,3]. Metastatic involvement of skeletal muscle is rare.

Until recently, asymptomatic skeletal muscle metastases (SMM) were detected only fortuitously. The widespread use of positron emission tomography/computed tomography (PET/CT) resulted in the detection of asymptomatic metastases in different organs, including skeletal muscle. A number of case reports or case series on SMM in patients with different primary tumors, including esophageal carcinoma, have been published[4-8], but the natural history of SMM in patients with esophageal carcinoma remains largely unknown. We report here five cases of patients with esophageal carcinoma and SMM observed in our center.

CASE REPORT

Records of 205 patients with esophageal carcinoma treated at our center who had PET/CT between 2006 and 2010 were searched to identify patients who had SMM diagnosed.

SMM were diagnosed in 5 patients (2%), all males. In one of these patients, SMM were subsequently shown to be related to a second primary tumor (Table 1). The details of these cases are outlined below.

Case 1

A 64-year-old male was diagnosed with adenocarcinoma of the distal esophagus in February 2004. The staging examination that included laparoscopy did not reveal distant metastases. The patient was treated with neoadjuvant chemoradiation. Transhiatal esophagectomy was performed on June 1, 2004. Histological examination of the resection specimen revealed pathologic complete response. The course of the follow-up was uneventful until October 2006 when the patient observed a resistance in the left thigh. Whole body [^{18}F] fluorodeoxyglucose (FDG) PET/CT performed in December 2006 revealed high focal uptake in the left quadriceps femoris muscle and in the left inguinal and iliac lymph nodes (Figure 1), with no evidence of a second primary tumor. An excisional biopsy established the diagnosis of SMM of adenocarcinoma.

The metastatic involvement of skeletal muscle and lymph nodes was treated with external beam radiation (50 Gy) that was administered in February and March 2007 along with 2 cycles of systemic chemotherapy (cisplatin and 5-fluorouracil). Four cycles of systemic chemotherapy using the same schedule have been administered subsequently until September 2007. A control FDG PET/CT in October 2007 demonstrated persistent uptake of radioactivity in the thigh metastasis that decreased in size. Additional metastatic lesions were discovered in medial condylus of the left femur and the retroperitoneal lymph nodes. The patient was feeling well and was subsequently only followed. In May 2008 an edema of the left lower extremity manifested. Control FDG PET/CT in June 2008 demonstrated uptake in retroperitoneal, mediastinal and left supraclavicular lymph nodes, lungs and left tibia as well as persistent accumulation in the left thigh. Between July and October 2008, the patient was treated with 5 cycles of combination of cisplatin and 5-fluorouracil. After the completion of chemotherapy, only symptomatic therapy was administered. The patient died on May 18, 2009, 31 mo after the diagnosis of skeletal muscle metastasis. An autopsy was not performed.

Case 2

76-year-old male was diagnosed in June 2006 with squamous cell carcinoma of the middle esophagus. Staging FDG PET/CT did not reveal distant metastases or evidence of a second primary tumor. The patient was treated with neoadjuvant chemoradiation that consisted of external beam radiation (50 Gy in 25 fractions of 2 Gy with systemic cisplatin and 5-fluorouracil). The control FDG PET/CT in November 2006 revealed complete disappearance of FDG uptake in the primary, but a pathologic accumulation in the right gluteus minimus muscle (21 mm × 14 mm in size) was noted. Because of complete response, patient age and uncertainty about the systemic disease, esophagectomy was not performed, and the patient was further only followed. On control FDG PET/CT, no uptake in the primary tumor was observed, but the size of the lesion in gluteus minimus muscle increased to 66 mm × 44 mm × 34 mm and involved bone. In addition, similar lesion was noted in the left psoas muscle. A biopsy performed in April 2007 confirmed metastasis of squamous cell carcinoma. Between May and July 2007 external beam radiation was administered to both lesions (70 Gy in 35 fractions of 2 Gy) concomitantly with 2 cycles of systemic cisplatin and 5-fluorouracil. Control FDG PET/CT in September 2007 demonstrated metastatic spread to the lungs, pleura, adrenals and retroperitoneal lymph nodes. Only symptomatic therapy was administered after the diagnosis of progressive metastatic disease, and the patient died on March 4, 2008, 16 mo after detection of SMM. An autopsy was not performed.

Case 3

A 57-year-old male was diagnosed in September 2006 with squamous cell carcinoma of the middle third of the esophagus. Staging FDG PET/CT performed in October 2006 revealed an accumulation of radioactivity not only in the primary tumor, but also in the skeleton of the pelvis and in the right subscapularis muscle. No evidence of a second primary tumor was found. The biopsy of the lesions was considered technically difficult, and the diagnosis of metastatic esophageal carcinoma was made based on multiplicity and appearance of lesions. Between October and December 2006 the patient was treated with 3 cycles of systemic chemotherapy (cisplatin and 5-fluorouracil). Chemotherapy had to be interrupted in January 2007 because of pneumonia. A control FDG PET/CT

Table 1 Patients with esophageal carcinoma and skeletal muscle metastases in the present series

Patient	Age at diagnosis (yr)	Histology	Stage at diagnosis	Timing	Primary therapy	Metastatic interval (mo)	Symptoms	Diagnosis	Location	Other metastases	Therapy for muscle metastases	Survival from the diagnosis of muscle metastasis (mo)
1	64	Adeno	Ⅱ	Metachronous	CXRT	32	Mass	PET/CT; biopsy	Quadriceps femoris	B; LN (subs)	CXRT	31
2	76	SCC	Ⅲ	Metachronous	CXRT	5	None	PET/CT; biopsy	Gluteus minimus; psoas	L; P; A; LN (subs)	CXRT	16
3	57	SCC	Ⅳ	Synchronous	None	0	None	PET/CT	Subscapularis	B	CRT; subs XRT	5
4	42	Adeno	Ⅳ	Synchronous	CXRT	0	None	PET/CT; biopsy	Iliacus	PR	S	16
5	60	SCC; adeno (pancreas)	Ⅳ	Synchronous	None	0	None	PET/CT; autopsy	Multiple	Multiple	None	2

A: Adrenal; Adeno: Adenocarcinoma; B: Bone; CRT: Chemotherapy; CXRT: Chemoradiation; L: Lungs; P: Pleura; PR: Peritoneum; LN: Lymph nodes; S: Surgery; SCC: Squamous cell carcinoma; subs: Subsequently; XRT: Radiotherapy; PET/CT: Positron emission tomography/computed tomography.

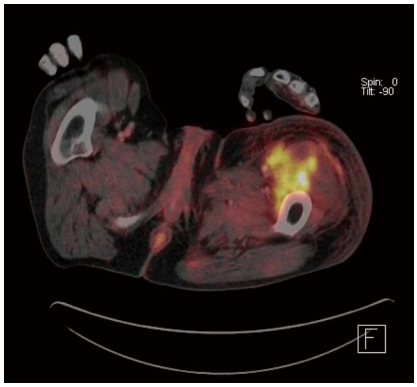


Figure 1 [^{18}F] fluorodeoxyglucose positron emission tomography/computed tomography showing a metastasis in the left quadriceps femoris muscle.

in February 2007 revealed mild progression. Palliative external beam radiation therapy (45 Gy in 18 fractions of 2.5 Gy) of all three sites of disease was then performed, but the condition of the patient deteriorated, with the progression of dysphagia. The patient died on March 24, 2007, 5 mo after the diagnosis of SMM. An autopsy was not performed.

Case 4

42-year-old male presented with adenocarcinoma of distal esophagus and cardia in April 2010. Staging FDG PET/CT demonstrated high FDG uptake in the primary tumor and a 2 cm mass in the iliacus muscle, with no evidence of a second primary tumor. As staging laparoscopy performed in May 2010 did not reveal spread in the abdominal cavity, the patient was referred for concomitant chemoradiation (50 Gy in 25 fractions of 2 Gy) and 2 cycles of systemic chemotherapy (cisplatin and 5-fluorouracil). Control PET/CT in October 2010 demonstrated only moderate progression in the primary, but marked progression of SMM (4 cm). The patient underwent subsequently (in October 2010) esophagectomy and resection of the muscle mass. Histologic examination of the muscle metastasis demonstrated poorly differentiated adenocarcinoma similar to the esophageal primary. The patient was then followed. In April 2011, the patient presented again with dysphagic complaints. Stenosis of the anastomosis was detected, and biopsy confirmed recurrence of adenocarcinoma. During the surgery to perform a jejunostomy, peritoneal carcinomatosis was detected and a large abdominal metastasis was resected. After a brief course of investigational therapy in a clinical trial the disease progressed and the patient was treated symptomatically. The patient died on August 27, 2011, 16 mo after diagnosis of SMM. An autopsy was not performed.

Case 5

60-year-old male was diagnosed in January 2010 with squamous cell carcinoma of the distal esophagus. Staging FDG PET/CT performed in February 2010 demonstrated high uptake in the tumor of distal esophagus, in lymph nodes adjacent to lesser curvature of the stomach, in the liver, left adrenal, bone and multiple metastases in skeletal muscle, including gluteus maximus muscle. Because of the extent of metastatic disease, only symptomatic therapy was indicated and histological verification of metastases was not sought. An esophageal stent was introduced to palliate the stenosis. The procedure resulted in the relief of the symptoms of short duration, but in March 2010 the condition of the patient deteriorated, he was admitted for back pain and died on March 21, 2010. Autopsy demonstrated, besides squamous cell carcinoma of the distal esophagus, a second primary pancreatic adenocarcinoma. Autopsy also revealed metastases in mediastinal and abdominal lymph nodes, peritoneum, pleura, lung, liver, kidneys, adrenal, thyroid, left submandibular gland, skeletal muscle, subcutis and bone. The histology of all metastases was adenocarcinoma pointing to pancreatic primary.

Table 2 Overview of the reports of esophageal carcinoma skeletal muscle metastases

Ref.	Sex	Age (yr)	Histology	Timing	Symptoms	Diagnosis	Location of muscle metastases	Other metastases	Therapy	Outcome
Heyer *et al*[18]	M	54	Adeno	Synchronous	Asymptomatic; fortuitously detected during CT	CT; MRI; biopsy	Multiple	None	CXRT (to the primary)	ND
Rehman *et al*[19]	M	71	Adeno	ND	Pain; unable to walk	MRI; biopsy	Thigh	ND	XRT (metastases)	Symptomatic relief 2 wk later
Kozyreva *et al*[20]	M	72	Adeno (T3N1Mx)	Synchronous	Asymptomatic; fortuitously detected during PET/CT	PET/CT; biopsy	Deltoid	None	C (oxaliplatin + 5FU/FA)	Progression of muscle metastases
Schultz *et al*[5]	ND	ND	SCC	Synchronous	ND	CT; biopsy	Gluteus minimus	ND	ND	ND
Heffernan *et al*[21]	F	67	Adeno	5 mo after surgery	Asymptomatic; fortuitously detected during PET/CT	PET/CT	Multiple	Mediastinum	ND	ND
Wu *et al*[22]	M	67	Adeno	Synchronous	Asymptomatic; fortuitously detected during PET/CT	PET/CT; biopsy	Gluteus minimus	None	ND	ND
Lekse *et al*[23]	F	78	Adeno	4 mo after diagnosis	Diplopia	CT; biopsy	Inferior rectus ocular	Lung	Symptomatic	ND
Lekse *et al*[23]	M	78	Adeno	2 mo after diagnosis	Diplopia; loss of vision	MRI	Lateral rectus ocular	Lung; bone; liver	XRT; C (5FU + FA)	ND

5FU: 5-fluorouracil; Adeno: Adenocarcinoma; C: Chemotherapy; CT: Computed tomography; CXRT: Chemoradiation; FA: Folinic acid; M: Male; F: Female; MRI: Magnetic resonance imaging; ND: Not described; PET: Positron emission tomography; XRT: Radiotherapy; SCC: Squamous cell carcinoma.

DISCUSSION

The 2% incidence of SMM in the present series may be affected by selection bias, as the primary diagnosis in one of the patients was performed before 2006. The information on esophageal carcinoma SMM is limited to individual case reports (Table 2), or to series of patients with SMM[5]. However, in most series of patients with SMM, no cases of patients with esophageal primary have been described[4,6-8]. SMM were also not reported in most autopsy series of esophageal carcinoma patients[2,3,9,10]. Cases of SMM have also been described in patients with gastric cancer[6]. To the best of our knowledge, only one case of squamous cell carcinoma of the esophagus metastatic to the skeletal muscle has been reported so far, with few details described[5]. Two of the patients reported here therefore represent the second description SMM of squamous cell carcinoma of the esophagus. The case of the patient with clinically undetected pancreatic adenocarcinoma demonstrates that histological verification may be needed not only to verify the neoplastic nature of the muscle lesion, but also to determine the primary as the frequency of second primary tumors is increasing. This case also indicates that a possible occult second primary tumor represents limitation in retrospective series of rare distant metastases. Histological verification confirming esophageal primary origin was possible in 3 out of 4 remaining cases. In all four cases SMMs were the first manifestation of metastatic disease. In patients with metachronous metastases, SMM involvement preceded the manifestation of metastatic spread to other organs by several months and survival times were relatively favorable in the context of metastatic esophageal carcinoma.

SMM detected with imaging studies were reported in 1%-2% of all patients with metastatic disease, with metastatic involvement limited to skeletal muscle reported in less than 10% of patients[11]. Similarly to the present series, most patients with SMM have metastasis only in one muscle[11]. Given the mass of skeletal muscle in the body, one would expect the metastases to this tissue to be more frequent. Experimental data suggest that muscle contractions result in mechanical destruction of tumor cells arrested in the microvasculature[12]. It has also been hypothesized that high lactate concentrations in the skeletal muscle microenvironment may decrease the responsiveness of muscle vessels to hypoxia, creating an environment unfavorable to angiogenesis[13].

PET/CT is currently being extensively utilized for pre-therapeutic staging of esophageal carcinoma because it was demonstrated that distant metastases missed by other imaging studies may be detected[14,15]. Individual cases of esophageal carcinoma SMM have been noted in cohorts of patients staged by PET/CT[16]. The increased incidence of esophageal carcinoma SMM may not only be the result of better diagnosis with PET/CT, but also of improved prognosis resulting from better control of the primary with the use of combined modality treatment. In fact, in one of the patients in the present series, the patient who had pathologic complete response after primary treatment two years earlier, the diagnosis could be made based on clinical presentation. It is therefore possible that with advances in the therapy of esophageal

carcinoma we will witness a further continuous increase of the incidence of SMM.

Little is known about the outcome of metastases affecting specific sites and optimal management of these patients, with the only source of information being anecdotal reports like the present series. In contrast to metastases affecting other organs, e.g., bone, no organ-specific drugs like bisphosphonates are available for SMM. Moreover, the potential utilization of new radiotherapy techniques in SMM is, for obvious reasons, limited. Prolonged survival has been observed in individual patients managed by combination of external beam radiation, surgery or chemotherapy[4,17]. Based on the experience in the present series and other cases reported in the literature, only general recommendations for the management of patients with SMM, including SMM of esophageal carcinoma, can be outlined. Important consideration is symptom control, and the therapeutic strategy has to be tailored according the muscle affected. Similarly to metastases of other location, the best control may be obtained with surgery which is, however, meaningful only in patients with isolated SMM. In addition, surgery may be necessary to establish the diagnosis of SMM. External beam radiation should be reserved for patients with inoperable or recurrent SMM, including patients with metastases in other sites, and best results may be obtained when combining radiation with platinum-based chemotherapy. No standard fractionation regimen or technique has been established in this rare indication. Outside of setting of chemoradiation regimens, the role of systemic chemotherapy in the management of SMM seems to be limited, but systemic chemotherapy may be an only option in patients with widespread metastases and good general condition. For patients in poor general condition, only symptomatic therapy (mostly corticosteroids and pain medication) is indicated.

In conclusion, while SMM represent a rare event in patients with esophageal carcinoma, the incidence may be increasing because of better diagnosis or improved control of the primary. SMM may represent the first manifestation of metastatic disease. An active therapeutic approach using surgery, external beam radiation and chemotherapy may result in survival that is substantially longer compared to other cases of metastatic esophageal carcinoma.

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CASE REPORT

Hepatic schwannoma: Imaging findings on CT, MRI and contrast-enhanced ultrasonography

Yu Ota, Kazunobu Aso, Kenji Watanabe, Takahiro Einama, Koji Imai, Hidenori Karasaki, Ryuji Sudo, Yosui Tamaki, Mituyoshi Okada, Yosihiko Tokusashi, Toru Kono, Naoyuki Miyokawa, Masakazu Haneda, Masahiko Taniguchi, Hiroyuki Furukawa

Yu Ota, Kazunobu Aso, Ryuji Sudo, Yosui Tamaki, Mituyoshi Okada, Masakazu Haneda, Division of Metabolism and Biosystemic Science, Department of Medicine, Asahikawa Medical University, Asahikawa 078-8510, Japan
Kenji Watanabe, Takahiro Einama, Koji Imai, Hidenori Karasaki, Toru Kono, Masahiko Taniguchi, Hiroyuki Furukawa, Division of Gastroenterologic and General Surgery, Department of Surgery, Asahikawa Medical University, Asahikawa 078-8510, Japan
Yosihiko Tokusashi, Naoyuki Miyokawa, Department of Pathology, Asahikawa Medical University, Asahikawa 078-8510, Japan
Author contributions: Ota Y contributed to this work mainly; Ota Y, Aso K, Watanabe K, Einama T, Imai K, Karasaki H, Sudo R, Tamaki Y, Okada M, Kono T, Haneda M, Taniguchi M and Furukawa H provided medical care; Ota Y, Aso K and Taniguchi M collected the images; Tokusashi Y and Miyokawa N diagnosed the patient pathologically; Ota Y and Taniguchi M wrote the paper.
Correspondence to: Masahiko Taniguchi, MD, Division of Gastroenterologic and General Surgery, Department of Surgery, Asahikawa Medical University, Asahikawa 078-8510, Japan. m-tani@asahikawa-med.ac.jp
Telephone: +81-166-682503 Fax: +81-166-682193
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Abstract

A primary benign schwannoma of the liver is extremely rare and is difficult to preoperatively discriminate from a malignant tumor. We compared the imaging and pathological findings, and examined the possibility of preoperatively diagnosing a benign liver schwannoma. A 72-year-old woman was admitted to our hospital because of a 4.6-cm mass in the liver. A malignant tumor was suspected, and a right hepatectomy was performed. After this, the diagnosis of a primary benign schwannoma of the liver was made through pathological examination. Contrast-enhanced ultrasonography (CEUS) with Sonazoid showed minute blood flows into the septum and solid areas of the tumor in the vascular phase; most likely due to increased arterial flow associated with infiltration of chronic inflammatory cells. In the postvascular phase, CEUS showed contrast defect of cystic areas and delayed enhancement of solid areas; most likely due to aggregation of siderophores. Because discriminating between a benign and malignant schwannoma of the liver is difficult, surgery is generally recommended. However, the two key findings from CEUS may be useful in discriminating ancient schwannoma by recognizing the hemorrhage involved in the secondary degeneration and aggregation of siderophores.

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Key words: Liver schwannoma; Contrast-enhanced ultrasonography; Liver resection

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Ota Y, Aso K, Watanabe K, Einama T, Imai K, Karasaki H, Sudo R, Tamaki Y, Okada M, Tokusashi Y, Kono T, Miyokawa N, Haneda M, Taniguchi M, Furukawa H. Hepatic schwannoma: Imaging findings on CT, MRI and contrast-enhanced ultrasonography. *World J Gastroenterol* 2012; 18(35): 4967-4972 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i35/4967.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i35.4967

INTRODUCTION

A schwannoma is a benign nerve sheath tumor that

originates from the Schwann cells of the nerve sheath. Schwannomas can occur in patients at any age, but are most commonly detected in patients between 20 years and 50 years of age. Although a schwannoma can develop in any part of the body, the most common sites include the head, neck, and flexor surfaces of the extremities[1]. Schwannomas develop infrequently in the gastrointestinal tract, the retroperitoneal cavity, and any other part of the body[2]. Thus, a primary benign schwannoma of the liver is extremely rare. Preoperative discrimination between a benign and malignant schwannoma is difficult; therefore, operation is typically recommended for a diagnosis. In the current study, we used a combination of computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET), but discrimination was still difficult. The use of preoperative imaging findings to detect liver schwannoma or the use of contrast-enhanced ultrasonography (CEUS) to detect and characterize focal liver lesions has not been previously undertaken. In this paper, we report a case of benign schwannoma, which we diagnosed using a novel method that combined CEUS with CT, MRI and PET imaging. In addition, we compared the imaging and pathological findings.

CASE REPORT

A 72-year-old woman was admitted to our hospital because a 4.6-cm mass was detected in her liver by ultrasonography (US) at a private clinic. Upon physical examination, the abdomen was soft and flat without tenderness. The liver, spleen, and superficial lymph nodes were not palpable. Blood analysis showed almost normal liver function. Carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) were both negative. Hepatitis B surface antigen and hepatitis B e antibody were positive, but hepatitis C virus marker was negative. The patient's serum antibodies were negative for specific echinococcal antigens.

CT, MRI, PET and CEUS were used for the qualitative diagnosis of the liver mass. Multiphasic contrast-enhanced CT in the arterial dominant, portal venous and late venous phases was performed using a 64-slice CT scanner. A CT scan of the abdomen showed an approximately 5-cm, well-defined round tumor in segment 8 of the right hepatic lobe. The mass was hypodense and contained a minute partial calcification (Figure 1A). The mass showed heterogeneous enhancement in the arterial dominant phase (Figure 1B) and delayed enhancement until the portal venous (Figure 1C) and late venous (Figure 1D) phases. There was no evidence of fatty infiltration or cirrhosis in the background liver.

MRI revealed a hypointense mass with T1-weighted imaging (Figure 2A) and a mixed hypo- and hyperintense mass with T2-weighted imaging (Figure 2B) and diffusion-weighted imaging (DWI) (Figure 2C). The diffusion images were obtained with a b value of 1000 s/mm^2. Because the mass was hyperintense on the apparent diffusion coefficient maps, it was also hyperintense on DWI under the influence of the T2 shine-through. Gadolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid (Gd-EOB-DTPA) was injected at 0.025 mmol/kg body weight. Gd-EOB-DTPA-enhanced MRI showed heterogeneous enhancement in the arterial phase and delayed enhancement until the late venous phase (similar to dynamic CT imaging), but a defect in the hepatobiliary phase (Figure 2D).

An F-18 fluorodeoxyglucose PET (FDG-PET) system was used (Discovery VCT; GE Healthcare, Tokyo, Japan) with the following conditions: PET, 2-3 min/1 bed, 3.75-mm slice; CT, 120 kV, 100 mA with auto mA; tube rotation time, 0.6 s/rotation; detector collimation, 64 mm × 1.25 mm; beam pitch, 0.984; section thickness, 5.0 mm; and reconstruction pitch, 3.27 mm. FDG-PET revealed abnormal uptake in the lesion, with a maximum standardized uptake value of 3.9. There was no additional lesion in the liver, no evidence of extrahepatic disease, and no lymphadenopathy.

US showed a 4.6-cm mass that contained multiple hypoechoic cysts with mixed internal septations and solid areas (Figure 3A). CEUS with Sonazoid showed minute blood flows into the septum and solid areas of the tumor in the vascular phase (Figure 3B), and contrast defects of cystic areas and delayed enhancement of solid areas in the postvascular phase (Figure 3C).

A malignant tumor, such as a biliary cystadenocarcinoma or cholangiocellular carcinoma (CCC), was suspected. Because there was danger of seeding, we did not perform a biopsy. An operation was performed to remove the mass. At surgery, it was discovered that the tumor originated from the anterior segment of Glisson's sheath, but was tightly attached to the posterior segment of the sheath. Intraoperative cholangiography revealed a stricture in the posterior segment of the bile duct, and there was no extension of the distal bile duct. The bile duct did not have a tumor projection until strangulation. Because the tumor involved the bile duct, dissection was not possible; therefore, the patient underwent right hepatectomy.

The gross findings were a well-circumscribed, whitish/brownish, localized mass, 4.5 cm in size, which was surrounded by a fibrous capsule. The division surface of the tumor showed large and small multilocular cysts. Microscopic examination revealed that the tumor was adjacent to the peripheral nerve of the portal area, and the Antoni A hypercellular area and Antoni B hypocellular area were present in a complex (Figure 4A). In the Antoni A region, an array of irregular groups of spindle cells was seen, but nuclear palisading with Verocay bodies was not observed. In the Antoni B region, there was edematous stroma, vasodilatation, aggregation of siderophores, and slight permeation of chronic inflammatory cells of the histiocyte and the lymphocyte types (Figure 4B). Immunohistochemical staining showed a strong positive S-100 protein reaction. Vimentin, nonspecific enolase, bcl-2, and MIC2 (also known as CD99) staining

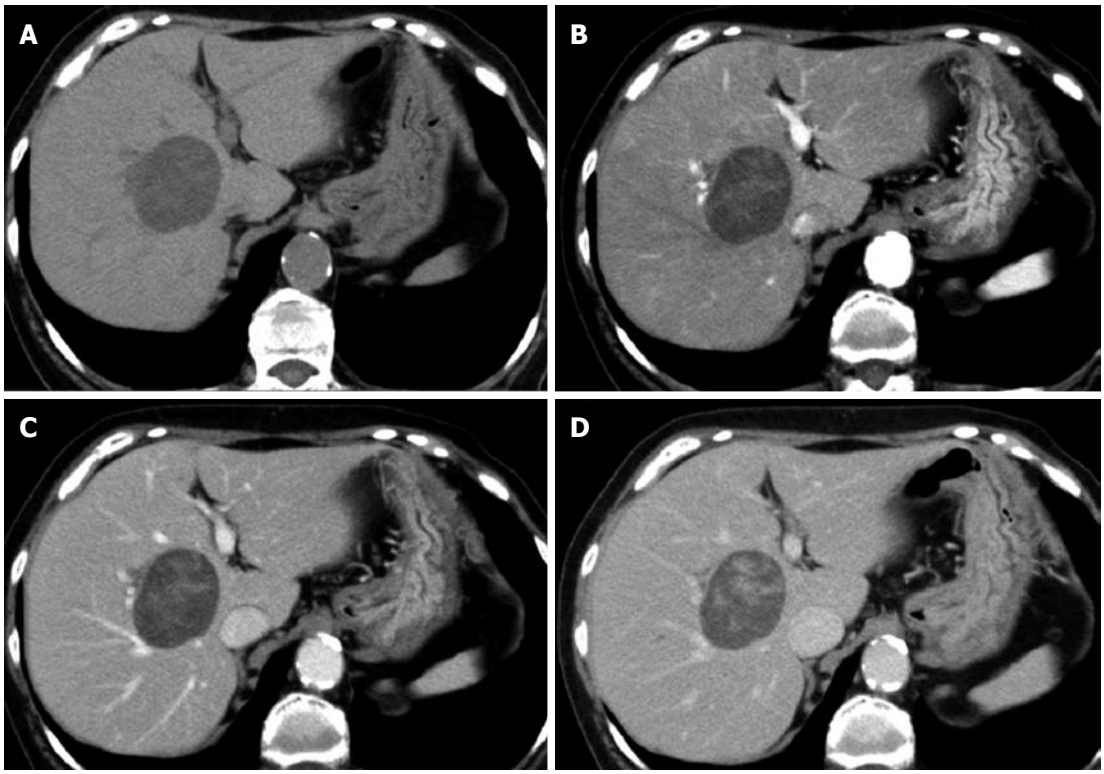


Figure 1 Abdominal computed tomography. A: Plain; B: Arterial dominant phase; C: Portal venous phase; D: Late venous phase. Abdominal computed tomography (CT) showed an approximately 5-cm well-defined round tumor in segment 8 of the right hepatic lobe. CT showed heterogeneous enhancement in the arterial dominant phase; and delayed the enhancement until the portal venous and late venous phases.

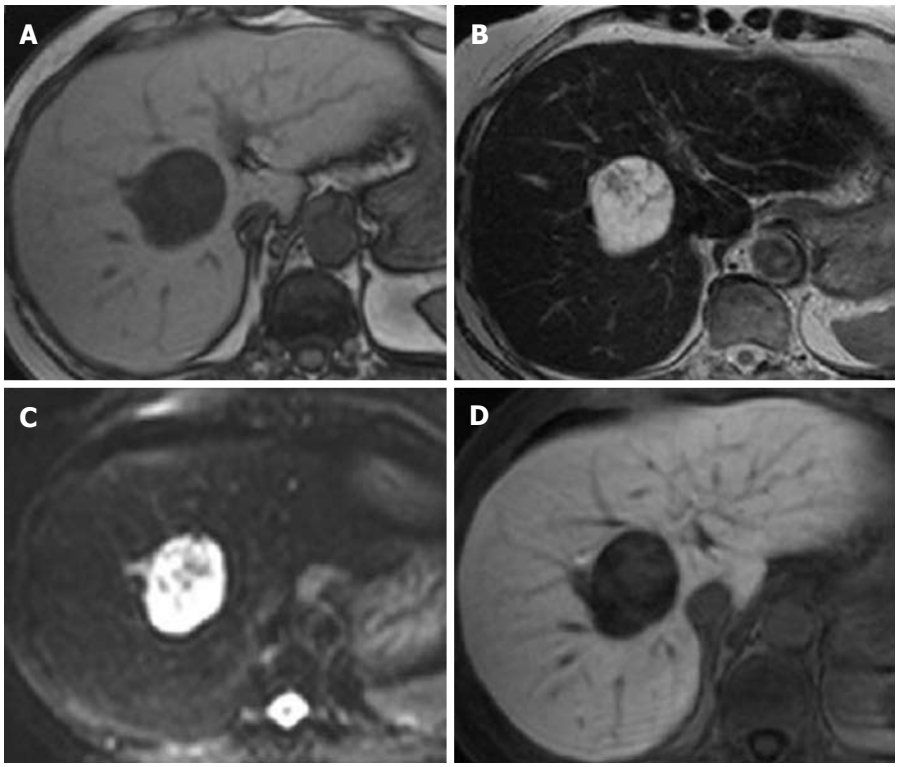


Figure 2 Magnetic resonance imaging. A: T1-weighted imaging; B: T2-weighted imaging; C: Diffusion-weighted imaging (DWI); D: Hepatobiliary phase. Magnetic resonance imaging (MRI) revealed a hypointense mass on T1-weighted imaging, and a mixed hypo- and hyperintense mass on T2-weighted imaging and on DWI. Gadolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid-enhanced MRI showed a defect in the hepatobiliary phase.

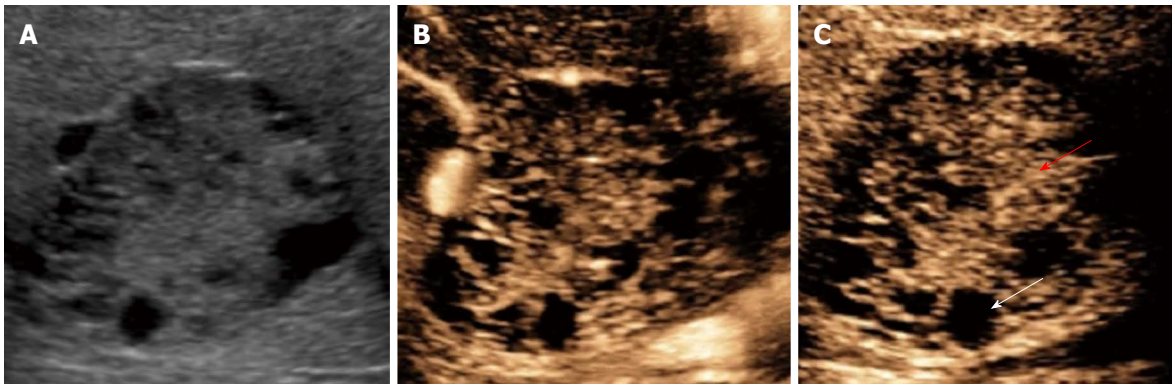


Figure 3 Contrast-enhanced ultrasonography with Sonazoid. A: B-mode ultrasound; B: Vascular phase; C: Postvascular phase. Ultrasonography showed a 4.6-cm mass that contained multiple hypoechoic cysts with mixed internal septations and solid areas. Contrast-enhanced ultrasonography with Sonazoid showed minute blood flow into the septum and solid areas of the tumor in the vascular phase, and contrast defect of cystic areas (white arrow) and delayed enhancement of solid areas (red arrow) in the postvascular phase.

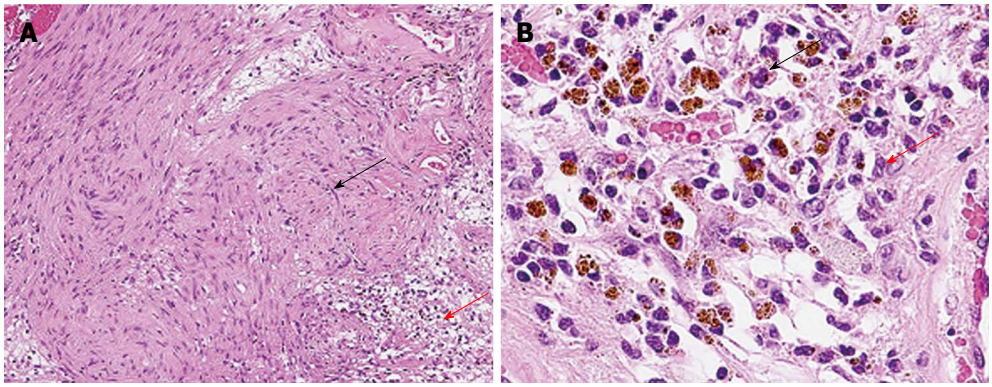


Figure 4 Microscopic examination. A: B-mode ultrasound. Microscopic examination revealed that Antoni A hypercellular area (black arrow) and Antoni B hypocellular area (red arrow) existed together in a complex; B: Vascular phase. In the Antoni B region, there were edematous stroma, vasodilatation, aggregation of siderophores (black arrow), and slight permeation of chronic inflammatory cells of the histiocyte and lymphocyte types (red arrow).

was also positive. The MIB-1 index was 2%. However, desmin, α-smooth muscle actin, CD34, c-kit, HMB-45, neurofilament, glial fibrillary acidic protein, and cytokeratin (AE1/AE3) staining was all negative.

On the basis of these findings, we diagnosed the patient as having a primary benign schwannoma of the liver. The patient was healthy and free from recurrence at a follow-up CT scan, which was performed 12 mo after surgery.

DISCUSSION

Histologically, schwannomas are encapsulated tumors that arise within nerve sheaths and consist of a highly ordered cellular component (Antoni A area) and a loose myxoid component (Antoni B area). In the subcellular component, spindle cells are present. Larger schwannomas have a tendency to undergo secondary degeneration, such as pseudocystic regression, hemorrhage, and calcification[3]. A primary benign schwannoma of the liver is an isolated benign tumor surrounded by a fibrous capsule. Immunohistochemical staining is diffusely and strongly positive for S-100 protein in a schwannoma, which is consistent with the markers for a nerve sheath tumor[3]. There are no specific characteristics with regard to the radiological appearance[1].

We reviewed the country of origin, size, age, sex, associated diseases, diagnosis, and prognosis of primary benign or malignant schwannomas; the findings are shown in Table 1[3-18]. Primary benign or malignant schwannomas were found in 14 women and 4 men. The age of onset was from 35 years to 74 years, and the mean age of onset was 57.5 years. While 13 cases were Asian in origin, there were no race differences. Although many hepatic schwannomas were Asian in origin, there has been no report of a causal relationship with hepatitis-associated viruses, and thus the reason for this correlation is unknown.

Generally, a CT scan depicts a schwannoma as a well-defined hypodense area, and enhanced CT shows peripheral enhancement, with an irregular pattern on the inside[10]. A delayed peripheral subject enhancement until the late venous phase in the CT scan reflects a fibrous capsule and an internal fibrillary element. With the aid of MRI, schwannomas have been described as masses with hypointensity on T1-weighted images and as masses with inhomogeneous hyperintensity or, in some cases, masses with internal heterogeneous mixed hypo- and hyperin-

Table 1 Review of primary benign or malignant schwannoma

Ref.	Age/sex	Country of origin	Size (cm)	Associated diseases	Diagnosis	Prognosis
Tuder *et al*[18]	74/M	North America	21 × 12 × 8	None	Surgery/autopsy	Death
Abe *et al*[17]	68/F	Asia	8.0 × 6.0 × 4.5	None	Surgery	Survival
Hytiroglou *et al*[16]	67/M	North America	13 × 11 × 10	None	-	-
Heffron *et al*[15]	38/F	North America	5	None	Surgery	Survival
Yoshida *et al*[14]	56/F	Asia	16	None	-	-
Morikawa *et al*[13]	63/M	Asia	20 × 13	None	Autopsy	Death
Sheikh *et al*[12]	35/F	Asia	-	None	-	-
Fiel *et al*[11]	49/M	North America	-	None	-	-
Wada *et al*[10]	69/F	Asia	15 × 13	None	Surgery	Survival
Wada *et al*[10]	64/F	Asia	4	Early gastric cancer	Surgery	Survival
Flemming *et al*[9]	57/F	Europa	Huge	None	Surgery	Survival
Momtahen *et al*[8]	52/F	Asia	4.4 × 3.6 × 2.9	None	Surgery	Survival
Lee *et al*[7]	36/F	Asia	5 × 4 × 2	None	Surgery	Survival
Akin *et al*[6]	66/F	Asia	3 × 5 × 4	Breast cancer	Biopsy/surgery	Survival
Shih *et al*[5]	58/F	Asia	9.1 × 7.1 × 8.9	None	Surgery	Survival
Sukegawa *et al*[4]	55/F	Asia	4.0 × 2.5 × 2.0	None	Surgery	Survival
Ozkan *et al*[3]	56/F	Asia	15 × 10 × 10	None	Surgery	Survival
Ota *et al* (present case)	72/F	Asia	4.6	None	Surgery	Survival

M: Male; F: Female.

Table 2 Comparison between preoperative imaging and pathological findings

Preoperative imaging technique		Characteristics	Pathology
Dynamic CT		Delayed peripheral subject enhancement	A fibrous capsule An internal fibrillary element
T2-weighted MRI		Internal heterogeneous mixed hypo- and hyperintensity	Secondary degeneration such as pseudocystic regression and hemorrhage
FDG-PET		Abnormal uptake	High cellular density (Antoni A area)
Ultrasonography	B mode	Mixed internal septations and solid areas	Division surface showed big and small multilocular cysts
	Vascular phase	Minute blood flows into septum and solid areas	Increased arterial vessel flows associated with infiltration of chronic inflammatory cells
	Postvascular phase	Delayed enhancement of solid areas	Aggregation of siderophores

CT: Computed tomography; MRI: Magnetic resonance imaging; FDG-PET: F-18 fluorodeoxyglucose-positron emission tomography.

tensity on T2-weighted images[8]. These findings reflect secondary degeneration, such as pseudocystic regression and hemorrhage. Abnormal uptake in FDG-PET reflects the high cellular density of schwannomas[19] (Table 2).

In this study, CT and MRI showed a tumor containing multiple cysts. Therefore, echinococcosis, hepatobiliary cystadenoma/adenocarcinoma or cystic liver metastasis of epithelial tumor, angiomyolipoma, leiomyoma, solitary fibrous tumor, schwannoma, or neurofibroma of the mesenchyma were possible differential diagnoses. However, it is very difficult to diagnose a schwannoma preoperatively based on its radiological appearance. Furthermore, abnormal uptake in FDG-PET did not aid the diagnosis.

Therefore, we used CEUS with Sonazoid for the preoperative diagnosis. Mixed internal septations and solid areas in B mode were reflected faithfully in the gross findings of a division surface with large and small multilocular cysts. CEUS showed minute blood flows into the septum and solid areas of the tumor in the vascular phase. The start of an inflow into a node can be confirmed within 18 s after the administration of Sonazoid. We accordingly determined that the minute blood flows were derived from the hepatic artery. Increased arterial flows were observed with the infiltration of chronic inflammatory cells of the histiocyte and lymphocyte types.

CEUS showed a contrast defect in the cystic areas and a delayed enhancement of the solid areas in the postvascular phase, which may reflect aggregation of siderophores. For example, in the case of echinococcosis, hepatobiliary cystadenoma/adenocarcinoma, cystic metastases, or CCC, CEUS shows contrast defects of the entire tumor in the postvascular phase (Table 2). Therefore, the two findings in CEUS (minute arterial flows into the tumor along the nodal septum in the vascular phase and contrast defects of the cystic areas and delayed enhancement of solid areas in the postvascular phase) are potentially useful for diagnosing an ancient schwannoma by recognizing the hemorrhage involved in the secondary degeneration and aggregation of siderophores.

The discrimination of a benign or a malignant schwannoma is difficult with preoperative imaging. However, some findings may indicate the presence of rare neoplasms such as a malignant schwannoma. The radiological features of hepatic malignant schwannoma include a well-demarcated, hypodense mass on CT, non-enhancement

on enhanced CT, and hypovascularity on angiography; in particular, hypovascularity may be an important finding for diagnosis, because most malignant hepatic tumors are hypervascular. Another important finding is the high level of alkaline phosphatase in serum with normal serum levels of hepatic transaminase and tumor markers, including CEA and AFP[13]. Nonetheless, because discrimination between a benign and malignant schwannoma is difficult, surgery is typically recommended.

In cases of malignant schwannoma, there are no reported chemotherapies and the prognosis is poor. Because the schwannoma may be tightly attached to Glisson's sheath (as in this case), surgical removal of the tumor could be very difficult, and hepatectomy that includes Glisson's sheath could be required.

In conclusion, here, we report a case of benign schwannoma, which we diagnosed using a novel method that combined CEUS with CT, MRI and PET. Because it is difficult to discriminate between a benign or malignant schwannoma of the liver, surgery is generally recommended for such tumors. Our findings with CEUS revealed minute arterial flow into the tumor along the nodal septum in the vascular phase, and delayed enhancement of solid areas in the postvascular phase. These two key observations may be useful in diagnosing an ancient schwannoma by recognizing the hemorrhage involved in the secondary degeneration and aggregation of siderophores.

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Bruno Annibale, Associate Professor of Gastroenterology, School of Medicine II, University Sapienza, Sant' Andrea Hospital, Roma 00189, Italy

Magdy El-Salhy, MD PhD, Professor, Chief Gastroenterologist, Section for Gastroenterology, Department of Medicine, Stord Helse-Fonna Hospital, Box 4000, 54 09 Stord, Norway

Diego Garcia-Compean, MD, Professor, Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, México

Rasmus Goll, MD, PhD, Department of Gastroenterology, Clinic of Internal Medicine, University Hospital of North Norway, Sykehusveien, N-9038 Tromso, Norway

Kazuhiro Hanazaki, MD, Professor and Chairman, Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okohcho, Nankoku, Kochi 783-8505, Japan

Khaled Jadallah, MD, Assistant Professor of Medicine**, Consultant, Gastroenterologist and Hepatologist,** Department of Internal Medicine, King Abdullah University Hospital, Jordan University of Science and Technology, Irbid 22110, Jordan

Eric R Kallwitz, MD, Assistant Professor, Department of Medicine, University of Illinois, 840 S Wood Street, 7th Floor, MC 787, Chicago, Il 60612, United States

Dr. Cuneyt Kayaalp, MD, Professor, Department of General Surgery, Staff Surgeon of Gastrointestinal Surgery, Turgut Ozal Medical Center, Inonu University, Malatya 44315, Turkey

Richard A Kozarek, MD, Executive Director, Digestive Disease Institute, Virginia Mason Medical Center 1100 Ninth Avenue, PO Box 900, Seattle, WA 98111-0900, United States

Spiros Ladas, Professor, 1st Department of Internal Medicine-Propaedeutic, Medical School, Athens University, "Laiko" General Hospital, Agiou Thoma 17, 11527 Athens, Greece

John S Leeds, Consultant Gastroenterologist, Department of Gastroenterology, Room 2.39, Ashgrove House, Aberdeen Royal Infirmary, Foresterhill Road, Aberdeen, AB25 2ZN, Scotland, United Kingdom

Yu-Yuan Li, Professor, Department of Gastroenterology, First Municipal People's Hospital of Guangzhou, 1 Panfu Road, Guangzhou 510180, Guangdong Province, China

Greger Lindberg, MD, PhD, Department of Gastroenterology and Hepatology, Karolinska University Hospital, K63, SE-14186 Stockholm, Sweden

Satoru Motoyama, MD, PhD, Department of Surgery, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan

CS Pitchumoni, Professor, Robert Wood Johnson School of Medicine, Robert Wood Johnson School of Medicine, New Brunswiuc, NJ D8903, United States

Dr. Orhan Sezgin, Professor, Gastroenteroloji Bilim Dalı, Mersin Üniversitesi Tıp Fakültesi, 33190 Mersin, Turkey

Ron Shaoul, MD, Director, Pediatric Gastroenterology and Nutrition Unit, Meyer Children's Hospital, Rambam Medical Center, PO Box 9602, 31096 Haifa, Israel

Bruno Stieger, Professor, Department of Medicine, Division of Clinical Pharmacology and Toxicology, University Hospital, 8091 Zurich, Switzerland

Masahiro Tajika, MD, PhD, Department of Endoscopy, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan

Yoshitaka Takuma, MD, PhD, Department of Gastroenterology, Kurashiki Central Hospital, 1-1-1 Miwa, Kurashiki, Okayama 710-8602, Japan

Bobby Tingstedt, MD, PhD, Department of Surgery, University Hospital in Lund, Getingevägen 4, S-221 85 Lund, Sweden

Liang-Shun Wang, MD, Professor, Vice-superintendent, Shuang-Ho Hospital, Taipei Medical University, No.291, Jhongjheng Rd., Jhonghe City, New Taipei 237, Taiwan, China

Dr. Thomas Wex, PD, Clinic of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Magdeburg, Leipziger Str. 44, 39120 Magdeburg, Germany

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
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Chaoyang District, Beijing 100025, China
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http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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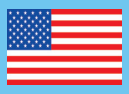
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Editorial Board Member of *World Journal of Gastroenterology*, Ioannis Kanellos, Professor, 4th Surgical Department, Aristotle University of Thessaloniki, Antheon 1, Panorama, 55236 Thessaloniki, Greece

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EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
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EDITORIAL

Pancreatic ductal adenocarcinoma screening: New perspectives

Raffaele Pezzilli, Dario Fabbri, Andrea Imbrogno

Raffaele Pezzilli, Dario Fabbri, Andrea Imbrogno, Department of Digestive Diseases and Internal Medicine, Sant'Orsola-Malpighi Hospital, 40138 Bologna, Italy
Author contributions: Pezzilli R, Fabbri D and Imbrogno A collected the literature data; Pezzilli R analyzed the data and interpreted the results; and Pezzilli R designed the study and wrote the manuscript.
Correspondence to: Raffaele Pezzilli, MD, Department of Digestive Diseases and Internal Medicine, Sant'Orsola-Malpighi Hospital, 40138 Bologna, Italy. raffaele.pezzilli@aosp.bo.it
Telephone: +39-51-6364148 Fax: +39-51-6364148
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Abstract

Pancreatic ductal adenocarcinoma accounts for more than 90% of all pancreatic cancers and its incidence has increased significantly worldwide. Patients with pancreatic ductal adenocarcinoma have a poor outcome and more than 95% of the people affected die from the disease within 12 mo after diagnosis. Surgery is the first-line treatment in the case of resectable neoplasm, but only 20% of patients are candidates for this approach. One of the reasons there are few candidates for surgery is that, during the early phases of the disease, the symptoms are poor or non-specific. Early diagnosis is of crucial importance to improve patient outcome; therefore, we are looking for a good screening test. The screening test must identify the disease in an early stage in order to be effective; having said this, a need exists to introduce the concept of "early" ductal adenocarcinoma. It has been reported that at least five additional years after the occurrence of the initiating mutation are required for the acquisition of metastatic ability of pancreatic adenocarcinoma and patients die an average of two years thereafter. We have reviewed the most recent literature in order to evaluate the present and future perspectives of screening programs of this deadly disease.

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Key words: Pancreatic neoplasms; Study population; Prevention; Therapeutics

Peer reviewers: Dr. Steven Hochwald, MD, Associate Professor and Chief, Surgical Oncology, Department of Surgery, University of Florida, 1600 SW Archer Rd, PO Box 100109, Gainesville, FL 32610, United States; Kotaro Miyake, MD, PhD, Department of Surgery, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan

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INTRODUCTION

Pancreatic ductal adenocarcinoma accounts for more than 90% of all pancreatic cancers. Over the last thirty years, its incidence in Europe and the United States has increased significantly; it ranks tenth among the most common solid tumours and it is the fourth leading cause of cancer death[1]. In addition, patients with pancreatic ductal adenocarcinoma have a poor outcome; more than 95% of the people affected die from the disease within 12 mo after diagnosis[2]. There is also a lack of really effective therapy; surgery is the first-line treatment in case of resectable neoplasm, but only 20% of patients are candidates for this approach[3].

One of the reasons there are few candidates for surgery is that, during the early phases of the disease when there is greater possibility of radical surgical treatment, the symptoms are poor or non-specific. On the other hand, when the symptoms become clear, the prognosis is poor. Specifically, the majority of patients have some

disturbances only shortly before diagnosis or problems 6 mo or less before diagnosis (pain or jaundice), and only a small portion have problems more than 6 mo before diagnosis. Among the latter, some patients have anorexia and/or early satiety and/or asthenia (7-20 mo before pain or jaundice), others have a distaste for coffee and/or smoking and/or wine (7-20 mo before) and still others have diabetes (7-24 mo before) or acute pancreatitis (8-26 mo before)[4]. Thus, the United States Preventive Services Task Force has a grade D recommendation against routine screening for pancreatic cancer for asymptomatic adults using abdominal palpation, ultrasonography or serologic markers for various clinical motives: (1) there is a poor prognosis for those diagnosed with pancreatic cancer; (2) it is thought that the evidence for diet-based prevention of pancreatic cancer is limited and conflicting; and (3) only some experts recommend lifestyle changes which may help to prevent pancreatic cancer, such as stopping the use of tobacco products, moderating alcohol intake and eating a balanced diet with sufficient fruit and vegetables[5].

On the other hand, we encourage the search to continue; the do-nothing attitude must be abandoned. Early diagnosis would be of crucial importance in improving the course of the disease; therefore, we are looking for a good screening test. The screening test must identify the disease in an early stage in order to be effective; having said this, a need exists to introduce the concept of "early" ductal adenocarcinoma. Yachida *et al*[6] report the data generated by sequencing the genomes of seven pancreatic cancer metastases to evaluate the clonal relationships among primary and metastatic cancers. The authors found that clonal populations which give rise to distant metastases are represented within the primary carcinoma, but these clones are genetically evolved from the original parental, non-metastatic clone. A quantitative analysis of the timing of the genetic evolution of pancreatic cancer indicates that there is at least a decade between the occurrence of the initiating mutation and the birth of the parental, non-metastatic founder cell. Thus, at least five additional years are required for the acquisition of metastatic ability and patients die on an average of two years thereafter. This conclusion indicates that the concept of early diagnosis of ductal adenocarcinoma should be moved back several years as compared to current practice, and this renders the screening increasingly more complicated.

PAST KNOWLEDGE

Traditionally, serum markers have been used for the early diagnosis and screening of ductal pancreatic adenocarcinoma, similar to what happens in other cancers. The most widely used serum markers for pancreatic cancer are the carcinoembryonic antigen and carbohydrate antigen 19-9 (CA 19-9); although their use has been extensively evaluated in clinical practice, several studies have shown that both have a low specificity as well as a low sensitivity[7]. We found that no significant differences in serum CA 19-9 levels were observed between patients with jaundice and those without as well as between those who underwent pancreatic resection and those who were medically treated because of advanced disease[7]. In addition, we found that in the 50 patients with histological/cytological examination, the sensitivity of this marker was 57.4%, the specificity was 0%, the positive predictive value was 90.0% and the negative predictive value was 0%[7]. Finally, elevated serum CA 19-9 values were present in similar proportions in patients with (61.9%) and without jaundice (64.7%, $P = 1.000$) as well as in 58.1% of resected patients and in 68.3% of those medically treated because of advanced disease ($P = 0.459$)[7]. These data further support the conclusions of the two American Society of Clinical Oncology recommendations for the use of tumor markers in pancreatic cancer, suggesting that CA 19-9 determination is not recommended for use as a screening test for pancreatic cancer and the use of CA 19-9 testing alone is not recommended for use in determining operability or the results of surgery in pancreatic cancer[8]. The reason for which biochemical screening fails to detect pancreatic cancer is that the production of CA 19-9 becomes readily detectable only in the advanced stages of pancreatic ductal adenocarcinoma after the onset of clinical symptoms, and it would not provide opportunities for early detection and cure (for example, in detecting precancerous lesions such as pancreatic intraepithelial neoplasia or intraductal pancreatic mucinous neoplasia at the initial stages)[9]. In conclusion, it is now time to abandon the routine use of CA 19-9 determination when pancreatic cancer is suspected.

PRESENT KNOWLEDGE

Currently, screening strategy is based on the identification of genetic alterations and populations at risk in order to identify algorithms for prevention and treatment[10].

There are many syndromes associated with pancreatic cancer, such as familial pancreatitis (associated with mutations in the *PRSS1* and *SPINK1* genes)[11], Peutz-Jeghers syndrome and familial atypical multiple mole melanoma syndrome[12]. In these cases, the suggested screening techniques are endoscopic ultrasound (EUS) or abdominal magnetic resonance imaging (MRI)[13]. Another risk factor is the presence of chronic pancreatitis; in fact, approximately 5% of patients with chronic pancreatitis will develop pancreatic cancer over a 20-year period. In these cases, until the development of more sophisticated imaging procedures able to detect the presence of cancer in an altered gland affected by chronic involvement, a screening test is not recommended[14].

Similarly to other cancers, such as colon cancer, pancreatic adenocarcinoma may be present in the same family[12]. It should be pointed out that subjects from families with a history of pancreatic cancer have an inherited predisposition of developing the disease, and subjects having familial pancreatic cancer may have at least two first-

Table 1 Major molecular markers in pancreatic ductal adenocarcinoma and their roles in screening

Marker	Implication of mutation	Diagnosis
EGF/EGFR	Constitutive activation of mitogen-activated protein kinase pathway	Screening for the patients more likely to respond to EGFR inhibitors
TGF-A	Promotes metastasis	
	Brings about epithelial mesenchymal transition	
VEGF/VEGFR	Tumor cell invasion and migration promoting tumor progression	Good diagnostic tool especially in combination with Cx43
	Lymphangiogenesis and lymphatic metastasis	
FGF	Cell proliferation, migration, and invasion	Serum FGF-BP1 can be used as a marker for high-risk premalignant lesions
MMPs	Digestion of extracellular components	MMP-2 in pancreatic juice
	Cell dissociation and subsequent invasion	Serum MMP-9
		Plasma MMP-7 levels help to distinguish between PC and CP
miRNAs	Invasive capacity	Plasma miRNA profiling for blood-based biomarkers
	Metastasis and maintenance of cancer	

EGF: Endothelial growth factor; EGFR: Endothelial growth factor together with its receptors; TGF-A: Tumor growth factor-A; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor and its receptor; FGF: Fibroblast growth factor; MMPs: Metalloproteinases; PC: Pancreatic carcinoma; CP: Chronic pancreatitis.

degree relatives with this disease, probably demonstrating autosomal dominant transmission[15]. A computer-based risk assessment tool has recently been developed and it has been shown to provide an accurate risk assessment for relatives with familial pancreatic cancer[16]. In addition, several studies have been carried out in patients with familial pancreatic cancer[17-23]; screening relatives from families in which pancreatic cancer is familial has a significant diagnostic yield, particularly in relatives > 65 years of age, confirming prior studies showing that magnetic resonance cholangiopancreatography as an initial screening modality is safe and effective[23].

One of the few currently known pre-neoplastic lesions is that of an intraductal papillary mucinous neoplasm (IPMN). If this lesion is located in the main duct (IPMN types 1 and 3), the treatment is surgical resection; on the other hand, if the lesion is located in the branch duct (IPMN type 2), the treatment depends on the size; if the diameter is less than 1 cm, therapeutic monitoring with MRI or CT scan once a year is sufficient. Monitoring is indicated every 6-12 mo if the diameter is between 1 and 2 cm; lesions between 2 and 3 cm should be monitored every 3-6 mo while, if the lesion is greater than 3 cm, or it is smaller but with the presence of high risk stigmata or symptoms, surgery is indicated[24]. Nevertheless, the diagnosis of IPMNs is usually incidental and therefore, a screening strategy in the general population is not possible; in fact, clinical symptoms are often vague or absent, and there is rarely an increase of serum amylase and lipase[25].

FUTURE PERSPECTIVES

The future of screening for pancreatic ductal adenocarcinoma is represented by the OMICs (genomic, proteomic, metabolomic). In particular, with regard to genomic, there are two studies which correlate the genetic alterations of pancreatic cells and the genetic alterations in the genome of the cells of the immune system. Baine *et al*[26] have studied the peripheral blood mononuclear cells from pancreatic cancer patients and matched healthy controls, analyzing them by means of whole genome cDNA microarray. They found that 383 genes were significantly different between pancreatic cancer patients and healthy controls, and 65 had at least a 1.5 fold change in expression. Pathway analysis revealed that many of these genes fell into the pathways responsible for hematopoietic differentiation, cytokine signaling, and natural killer cell and CD8+ T-cell cytotoxic response. Unsupervised hierarchical clustering analysis identified an eight-gene predictor set, consisting of *SSBP2*, *Ube2b-rs1*, *CA5B*, *F5*, *TBC1D8*, *ANXA3*, *ARG1* and *ADAMTS20* capable of distinguishing pancreatic cancer patients from healthy controls with an accuracy of 79% (sensitivity 83% and specificity 75%). In the future, these data may help in diagnosing pancreatic cancer earlier. In the same way, De Monte *et al*[27] have identified the complex interaction involving cells, such as stromal and immune cells, and tumor cells in the tumor microenvironment. The result of this is an alteration of the T-cells which, instead of producing cytokines effective in fighting cancer, produce cytokines capable of promoting further progression of the disease; this lymphocyte derangement has been called "deviant lymphocytes" by the authors. The authors also identified the molecules involved in this mechanism which will allow the development of therapies to curb this activity. Antibodies are already available for some of these molecules and they are capable of blocking their activity. Most important, in a series of patients undergoing surgery, the authors also showed the existence of a correlation between the quantity of "deviant cells" present in the tumor and patient survival showing the possibility of stratifying pancreatic cancer patients into two categories having a better or a worse prognosis.

The other aspect of the future of screening for pancreatic ductal adenocarcinoma is the proteomic; in particular, it is interesting to evaluate studies with large populations whether (or not) cancer cells secrete proteins whose levels rise early in serum or in pancreatic juice.

Presently, as shown in the Table 1, the major molecular markers in pancreatic ductal adenocarcinoma evaluated in clinical practice are the following: endothelial growth

factor together with its receptors (EGFR) involved in the activation of the mitogen-activated protein kinase pathway and used as a screening test for patients who are that more likely to respond to EGFR inhibitors: (1) tumor growth factor-A, which promotes metastasis and brings about epithelial mesenchymal transition; (2) vascular endothelial growth factor and its receptor, which indicate tumor cell invasion and migration promoting tumor progression, lymphangiogenesis and lymphatic metastasis and seem to be a good diagnostic tool, especially in combination with Cx43; (3) fibroblast growth factor, which is involved in cell proliferation, migration and invasion, and can be used as a marker for high-risk premalignant lesions; (4) metalloproteinases, which are markers of the digestion of extracellular components, cell dissociation and subsequent invasion found in the pancreatic juice, in serum or in plasma and help to distinguish between pancreatic adenocarcinoma and chronic pancreatitis; and (5) miRNAs which represent a marker of invasive capacity, metastasis and maintenance of cancer, and may be an aid for developing blood-based biomarkers[28]. However, the clinical studies are largely disappointing regarding the routine use of these markers[29-31].

Finally, it should be remembered that the last OMIC is metabolomic. Neoplastic cells have an accelerated metabolism; this results in an increase of the concentration of some biochemical components in serum, and these changes may be used as markers of pancreatic cancer. Bathe *et al*[32] have studied the variations of these biochemical components in patients with pancreatic cancer. In the pancreatic cancer group, as compared to benign cases, the serum concentrations of glutamate and glucose were the most elevated on multivariate analysis. In benign cases, creatine and glutamine were most abundant. To examine the usefulness of this test, a comparison was made to age- and gender-matched controls with benign lesions which mimicked cancer, also checking for the presence of jaundice and diabetes; the results were encouraging because the metabolic profile in patients with pancreatic cancer was distinguishable from patients with benign pancreatic lesions.

In conclusion, the primary prevention of pancreatic cancer is based on the assessment and elimination of major risk factors: smoking, high fat diet, diet rich in meat and low in vegetables and folate, occupational exposure, and obesity[33,34]. Presently, secondary prevention is limited to patients at risk for familial cancer and a screening plan should be established based on the implementation of an annual MRI or EUS.

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TOPIC HIGHLIGHT

Marcela Kopacova, MD, PhD, Associate Professor, *Series Editor*

Hepatorenal syndrome

Jan Lata

Jan Lata, Department of Internal Medicine, University Hospital and Faculty of Medicine, Ostrava University, 70103 Ostrava, Czech Republic
Author contributions: Lata J contributed solely to this work.
Correspondence to: Jan Lata, Professor, MD, PhD, Department of Internal Medicine, University Hospital and Faculty of Medicine, Ostrava University, Dvorakova 7, 70103 Ostrava, Czech Republic. jan.lata@osu.cz
Telephone: +420-59-7091013 Fax: +420-59-6118219
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Abstract

Hepatorenal syndrome (HRS) is defined as a functional renal failure in patients with liver disease with portal hypertension and it constitutes the climax of systemic circulatory changes associated with portal hypertension. This term refers to a precisely specified syndrome featuring in particular morphologically intact kidneys, where regulatory mechanisms have minimised glomerular filtration and maximised tubular resorption and urine concentration, which ultimately results in uraemia. The syndrome occurs almost exclusively in patients with ascites. Type 1 HRS develops as a consequence of a severe reduction of effective circulating volume due to both an extreme splanchnic arterial vasodilatation and a reduction of cardiac output. Type 2 HRS is characterised by a stable or slowly progressive renal failure so that its main clinical consequence is not acute renal failure, but refractory ascites, and its impact on prognosis is less negative. Liver transplantation is the most appropriate therapeutic method, nevertheless, only a few patients can receive it. The most suitable "bridge treatments" or treatment for patients ineligible for a liver transplant include terlipressin plus albumin. Terlipressin is at an initial dose of 0.5-1 mg every 4 h by intravenous bolus to 3 mg every 4 h in cases when there is no response. Renal function recovery can be achieved in less than 50% of patients and a considerable decrease in renal function may reoccur even in patients who have been responding to therapy over the short term. Transjugular intrahepatic portosystemic shunt plays only a marginal role in the treatment of HRS.

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Key words: Liver cirrhosis; Hepatorenal syndrome; Ascites; Vasoconstrictors; Terlipressin

Peer reviewer: Virendra Singh, MD, DM, Professor, Department of Hepatology, Graduate Institute of Medical Education and Research, Chandigarh 160012, India

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PATHOPHYSIOLOGY

The organic intactness of kidneys, a condition for the diagnosis of this syndrome, has been demonstrated repeatedly in terms of morphology as well as by normal function of kidneys from an individual with hepatorenal syndrome (HRS) transplanted to a person without any liver disease[1]. The main pathophysiological mechanisms include increased renal arterial resistance, especially affecting the cortex of kidneys, which results in renal hypoperfusion[2,3], and arterial hypotension. The small volume of the ultrafiltrate is reabsorbed almost completely in the proximal tubule whereas almost a zero quantity of sodium flows to the Henle's loop. As a result, enhanced aldosterone activity is of little application in this phase and standard diuretics have no effect either. Due to the adiuretin-vasopressin activity, final urine is produced through an essentially zero hyperosmolar natriuresis, and its quantity ranges between oliguric and anuric values. Under such conditions, the local prostaglandin regulation system, which is of little clinical significance other-

wise, comes to play a crucial role in the maintenance of residual renal perfusion, and its elimination (e.g., by non-steroidal antirheumatics) can have distinct consequences.

The principal mechanisms leading to renal vasoconstriction consist of alterations in systemic circulation, accompanying portal hypertension[4], which are represented by decreased peripheral vascular resistance with subsequent vasodilatation (a consequence of the hyperactivity of vasodilating agents), central hypervolemia, hyperkinetic circulation, and the activation of compensatory mechanisms, i.e., the sympathetic nervous system (SNS), renin-angiotensin-aldosterone system (RAAS) and adiuretin-vasopressin. What is more, adenosine, for example, which has a vasodilating effect in most tissues, produces vasoconstriction in kidneys[5]. The direct impact of the SNS and RAAS also stimulates the reabsorption of sodium in the proximal tubule[6].

It is important to point out that renal vasoconstriction itself is not sufficient for the development of hepatorenal syndrome. Arterial hypotension is the key factor which, even if it does not reach shock values, causes simultaneous renal vasoconstriction and renal hypoperfusion with decreased glomerular filtration[7]. Thus, cardiac output is the relevant factor. Cardiac output may be low, normal or high, but is relatively insufficient[8,9] to prevent a severe reduction of effective circulating volume due to the splanchnic arterial vasodilatation in patients with HRS. The reasons why cardiac output is relatively insufficient in end-stage liver disease is still unknown, but in recent years several specific cardiac abnormalities, such as reduced systolic and diastolic responses to stress stimuli, electrophysiological repolarisation changes or enlargement of cardiac ventricles, have been recognised as so-called "cirrhotic cardiomyopathy"[10].

In addition to this, other factors such as the release of endotoxins and a further release of biologically active substances such as inflammatory cytokines, nitric oxide, carbon monoxide and others as a result of a bacterial infection may further impair cardiac function in patients with end-stage liver disease.

The once considered theory of a direct reflex link between the liver (or the portal system) and renal circulation has been abandoned lately, although several experiments in the past managed to prove renal vasoconstriction (accompanied by renal function alterations in terms of sodium and water retention) as a result of portal (or intrasinusoidal) pressure elevation[11,12].

Because the above-mentioned circulatory alterations can virtually be observed from very early stages in diseases with portal hypertension, HRS has to be viewed as their climax, and the refractory ascites stage constitutes a significant turning point in such development. Nevertheless, there is surprisingly little connection to the progression of hepatic lesions; in other words, it cannot be assumed automatically that a patient with HRS has reached their terminal liver failure stage. In fact, a patient with a Child-Pugh Score of 8 or less has the same risk of HRS development as a patient in C class. In this respect, determination of sympathetic activity (plasma noradrenaline) or RAAS[13], or renal arterial resistance ("resistive index" in ultrasonography) has a considerably better prognostic value[14]. In principle, a high risk of hepatorenal failure must be expected in patients with liver disease and ascites, with an indication of dilution hyponatremia, with tachycardia and mean arterial pressure below 80 torr.

Consistent with the adopted diagnostic criteria, it would be suitable to differentiate between diagnoses of "hepatorenal syndrome" and "hepatorenal failure". In both cases, the clinical parameters of renal function are identical; however, the fundamental difference lies in the circumstances of their occurrence and prognosis. Hepatorenal syndrome means a precisely specified state of kidney function failure where all of its eliminable causes and precipitant factors have been excluded. "Hepatorenal failure" is a more general term which can be applied to any kidney function failure in liver disease with portal hypertension which adds a significant disposition to it. This implies that the presence of liver disease with liver function alterations and with portal hypertension is of crucial importance as well as the absence of an organic renal defect (proteinuria < 0.5 g/d, erythrocyturia < 50/field of view in high-resolution, normal ultrasound findings in kidneys and the excurrent duct system).

CLINICAL PRESENTATION

HRS occurs almost exclusively in patients with ascites. The occurrence probability in patients having an ascites for more than 5 years reaches 40%. Besides the above-mentioned laboratory prognostic factors, ascitic patients with a small liver, oesophageal varicose veins and an unbalanced diet have a higher risk of HRS incidence. Dilution hyponatremia, tachycardia and arterial hypotension with a mean arterial pressure of approximately 80 torr or less must be perceived as warning signs.

The prevalence of HRS in patients affected by liver cirrhosis with ascites is in effect equal to 18% after 1 year, rising to 39% at 5 years. In almost half of the cases of HRS, one or more precipitating factors can be identified, among which we can include bacterial infections (57%), gastrointestinal haemorrhage (36%) and therapeutic paracentesis (7%)[13].

According to the progression, or more precisely according to clinical seriousness, HRS can be classified in 2 types[15]: Type 1 - rapidly progressive, where the serum creatinine doubles in 2 wk and values of approximately 350 μmol/L (2.5 mg/dL) are usually achieved. This type accompanies clinically more serious conditions and it is typically unstable. Its main clinical feature is acute renal failure. Type 2 - slowly progressive, this state was described later and, despite the otherwise typical signs of hepatorenal failure, it is quite stable. Serum creatinine rises slowly or not at all and it usually does not exceed 180 μmol/L (1.3 mg/dL). The clinical record is dominated by refractory ascites and relatively stable liver function[16].

The difference in prognosis between the two types

of HRS is essential as the median survival of type 1 is about 2 wk while that of type 2 is generally around 4-6 mo[17]. Type 1 HRS is often induced by the occurrence of a precipitating stimulus. The important factor seems to be infections (e.g., urinary tract infections and infections of the biliary or intestinal tract)[18] but the most important thing is the development of a spontaneous bacterial peritonitis[19]. Almost one third of patients with spontaneous bacterial peritonitis develop a non-transient form of renal failure which in most cases fits the diagnostic criteria of type 1 HRS. The independent predictive factors for the development of renal failure as a consequence of bacterial infections are the severity of infection, the model for end-stage liver disease score at the diagnosis of infection and the lack of resolution of infection by means of antibiotics[20,21].

Up to the end of the last century, the prognosis for cirrhotic patients developing HRS was very poor. Thereafter, some new effective treatments of HRS which improve survival have been introduced with encouraging results.

DIAGNOSIS

There is no specific test to determine an unequivocal diagnosis of HRS. The prime finding consists of reduced glomerular filtration (creatinine clearance) < 40 mL/min or serum creatinine increase > 135 μmol/L under the exclusion of other causes of renal failure. The most relevant indications of the functional character of such failure include: natriuresis < 10 mmol/L, urine osmolality higher than plasma osmolality, natremia < 130 mmol/L and diuresis < 500 mL/d.

The criteria for diagnosing HRS have recently been re-examined by the International Ascites Club[22] (Table 1).

The main changes in the new diagnostic criteria of HRS as compared to those previously used[23] are the removal of minor diagnostic criteria and the removal of ongoing bacterial infection as an exclusion criterion for the diagnosis of HRS. Other important changes are that plasma volume expansion should no longer be performed with saline but with albumin, and creatinine clearance is no longer considered as a tool for diagnosis. The presence of shock, even of septic shock, as well as previous treatment with nonsteroidal anti-inflammatory drugs, other nephrotoxic drugs (i.e., aminoglycosides) and vasodilators (i.e., nitrates, prazosin and renin-angiotensin system inhibitors) are still considered exclusion criteria for the diagnosis of HRS, along with the presence of proteinuria, hematuria or ultrasound alterations of the kidneys.

In clinical practice, it is absolutely essential to examine urinalysis and sodium concentration in urine in order to differentiate organic renal insufficiency.

Certain diagnostic difficulties regarding differentiation may be the result of acute tubular necrosis which has been observed by approximately 5% of patients dying of renal failure. It is potentially reversible and is characterised by higher Na concentration in urine, isoosmolar urine, and the ratio of urine and plasma creatinine below 20:1. This disease often develops after the administration of a contrast medium to cirrhotic patients in computed tomography examination. Therefore, a very precise indication for use of computed tomography is required by such patients, or a substitution by magnetic resonance.

Table 1 New diagnostic criteria of hepatorenal syndrome

New diagnostic criteria of hepatorenal syndrome
Cirrhosis with ascites
Serum creatinine > 133 μmol/L (1.5 mg/dL)
No sustained improvement of serum creatinine (decrease to a level of 133 μmol/L or less) after at least 2 d of diuretic withdrawal and volume expansion with albumin; the recommended dose of albumin is 1 g/kg of body weight per day up to a maximum of 100 g/d
Absence of shock
No current or recent treatment with nephrotoxic drugs
Absence of parenchymal disease as indicated by proteinuria > 500 mg/d microhematuria (> 50 red blood cells per high-power field) and/or abnormal renal ultrasonography

Even the presence of hepatitis B and C virus can damage kidneys as it leads to membranous glomerulonephritis or vasculitis as well as chronic autoimmune liver disease. In such patients, renal failure may also be induced by cyclosporine treatment.

Finally, it is again worth pointing out that the risk of an organic renal defect tends to be higher in patients with advanced liver disease due to the impact of reduced blood perfusion of kidneys as well as alterations of immune and metabolic functions, and this needs to be taken into account.

TREATMENT

Although HRS is a terminal manifestation of portal hypertension in parenchymatous liver disease, it no longer ranks among the fundamentally insolvable issues. In specific cases, however, availability matters and the rational and ethical grounds for this rather costly treatment have to be considered.

Influence on causative factors

If there are indications of renal failure, first of all, the causative factors have to be eliminated and treated, such as: elimination of nephrotoxic medications; elimination or treatment of a suspect bacterial infection including spontaneous bacterial peritonitis with focus on gram-negative flora; elimination of bleeding in gastrointestinal tract and, if need be, adequate compensation for losses; elimination of nonsteroidal antirheumatics; elimination of diuretics (they enhance central hypervolemia and the sympathetic and RAAS activity); supplementation of intravascular volume, preferably through hypoalbuminemia correction (albumin is the most reliable volume expander with the longest lasting impact). Another effect, even if just transitory, may be brought about by the

partial evacuation of tension ascites accompanied by a consistent compensation for albumin. Efforts concerning hyponatremia substitution must be avoided (risk of cerebral oedema) - fluid restriction is more suitable.

Liver transplantation

Naturally, liver transplantation is the only rational solution in cases of advanced liver disease. However, the presence of a kidney function failure considerably aggravates the prognosis of such patients[24,25].

During the last century, only a few patients with HRS underwent liver transplantation, because most patients died before surgery as a consequence of the rapid evolution of type 1 HRS.

Novel treatment approaches, especially terlipressin plus albumin therapy, provide a "treatment bridge" towards liver transplantation. Treatment with terlipressin 4-6 mg/d for up to eight mo has been described, completed by successful liver transplantation[26,27]. The number of transplanted patients is still very low, however. In a prospective study, none of 15 type 1 HRS patients referred to a tertiary care transplant centre was transplanted: 12 patients had contraindications against transplantation and the remaining three patients died while awaiting transplantation[28].

Hypovolemia correction

With regards to the fact that the HRS laboratory finding is similar to prerenal uraemia, attempts have been made since the very beginning to influence this syndrome by means of hypovolemia correction. A physiological solution or Dextran has been administered, which is unfortunately inappropriate in this case. Nevertheless, human albumin has proven the most suitable. Nowadays, it is used for expansion of circulatory blood volume and has been shown to successfully reduce the incidence of HRS type 1. When used for treatment of HRS, it has shown better results when used in combination therapy as it amplifies the effect of other pharmacologic therapies in the treatment of HRS. Often, the quantity to be administered is high - up to 50 g/d. Anyway, hypovolemia correction is required before, as well as during, the subsequent treatment with medicaments[29] and albumin is now the basis of the therapy in combination with vasoconstrictors.

Vasoconstrictors

The first group of medicaments used for this indication in an effort to reduce intrarenal vascular resistance were prostaglandins. In spite of the fact they used to be recommended, they have not shown any provable improvement in renal function[30].

On the other hand, the application of systemic vasoconstrictors has been justified with respect to known pathogenetic factors. In HRS, marked reduction of effective circulating volume was found, which is related to a splanchnic arterial vasodilation and inadequate cardiac output, which implies an extreme overactivation of the endogenous systemic vasoconstrictor systems, i.e., the RAAS, SNS and nonosmotic release of vasopressin. This means that the final aim of the therapeutic approach is to reduce severe renal arterial vasoconstriction[51]. In the early years of treatment, dopamine was tested in a small to medium dose. Dopamine has been shown to reduce renal vascular resistance and increase renal blood flow. It was therefore thought to be potentially useful in the treatment of HRS and was tested in a small to medium dose. But its clinical effect has not been confirmed unequivocally[11], alone or even in combination with ornipressin[32].

Nonetheless, the application of synthetic analogues of vasopressin-terlipressin (N-triglycyl-8-lysine-vasopressin synthesised in the laboratories of the Czechoslovak Academy of Sciences in Prague, Czech Republic in 1964), or ornipressin represented a considerable step forward. Analogues act on the abundant V1 receptors in the splanchnic vasculature, causing greater vasoconstrictive effects in the mesenteric circulation than in renal or other vascular systems.

In retrospective studies, as well as in small prospective pilot studies, it has been demonstrated that prolonged use of an ornipressin[33,34], terlipressin[35-48] or α-agonist vasoconstrictor (midodrine plus octreotide, noradrenaline alone)[49-56] in association with human albumin is capable of recovering renal function in 40%-60% of patients with type 1 HRS. The use of ornipressin was, however, abandoned because of its high rate of ischemic side effects.

Currently, terlipressin is the preferred product. Treatment with terlipressin and albumin has become a major breakthrough in the field of cirrhosis[57]. It has been used in a broad range of HRS treatment applications and there are many studies demonstrating its efficiency. In 2006, The Cochrane Library published a review with the conclusion that terlipressin treatment was promising but its results had to be confirmed[58].

Nowadays, however, it has become the clearly preferred treatment in general recommendations and is the most widely used agent in the treatment of type 1 HRS. The terlipressin treatment protocol comprises an initial dose of 0.5-1 mg terlipressin applied by intravenous injection every 4-6 h or continuous intravenous infusion starting at an initial dose of 2 mg/d. Unless the creatinine level has dropped by 25% on the third day, the dose is raised to 2 mg every 4 h or 12 mg/d by continuous intravenous infusion, respectively. Intravenous albumin is administered in case of failure to maintain the central venous pressure at 10-15 cm H_2O (initially 1 g of albumin/kg for two days up to a maximum of 100 g/d followed by 20-40 g/d)[59].

The treatment continues until laboratory values have improved, otherwise no longer than 2 wk. Complete reversal (i.e., decrease of serum creatinine with a final value < 133 μmol/L) or partial reversal (defined with a decrease of serum creatinine > 50% with a final value ⩾ 133 μmol/L) of type 1 HRS has been observed in almost 59% of patients[60].

Usually, diuretics are not administered during therapy

with terlipressin and albumin; however, some surveys have used furosemide and albumin. Type 2 HRS is more frequent in clinical practice than type 1 and liver transplantation can cure this syndrome as well as other complications relating to hepatic insufficiency. Treatment efficiency has reached 80% in this indication. Nevertheless, recurrence is very common after discontinuation of treatment[61].

Recurrence of HRS after treatment withdrawal (i.e., a sharp increase in serum creatinine within a few days) occurs in approximately 20% of patients. Treatment with terlipressin should be repeated in these patients and this measure is frequently effective. This can result in long-term treatment (up to 8 mo) in some cases. The dose of terlipressin can be up to 4-6 mg/d and albumin up to 100 g/d[26,27,59].

An important question is prediction of response to this treatment. The most consistent predictor of response to terlipressin and of survival is the baseline serum creatinine, bilirubin and an increase in mean arterial pressure of $\geqslant$ 5 mm Hg at day 3 of treatment. Patients most likely to benefit from terlipressin have earlier onset renal failure [i.e., serum creatinine < 445 μmol/L (5.0 mg/dL)]. The cutoff level of serum bilirubin that best predicted response to treatment was 170 μmol/L (10 mg/dL). Improvement in the hyperdynamic circulation (measured by sustained rise in mean arterial pressure) is also important for reversal of HRS[62,63].

Other treatment alternatives, with a similar effect on circulatory parameters, may include alpha-adrenergic agonists. Their advantage compared to terlipressin is their lower price, but they seem to be less effective than terlipressin now. Several small studies have shown promise for norepinephrine in the treatment of HRS, however just one study showed a better (but not statistically significant) response to terlipressin.

Midodrine is an alpha-agonist and appears more convenient than norepinephrine. It offers the advantage of oral administration; however it was used in combination with octreotide (and albumin). It has been shown to moderately improve the systemic and renal hemodynamics.

Transjugular intrahepatic portosystemic shunt

Transjugular intrahepatic portosystemic shunt (TIPS) interferes with the hepatorenal reflex and brings about reduced sympathetic and RAAS activity through intensified hyperdynamic circulation. TIPS results in a positive effect on renal function, including HRS, demonstrated by a rapid increase in urinary sodium excretion, urinary volume, and improvement in plasma creatinine concentration. Its positive impact on renal function in liver cirrhosis with ascites has already been proven in several studies[64-67]. The first prospective survey with a well-documented type 1 HRS was published as early as 1998[68] and the effectiveness of TIPS has been reconfirmed since. TIPS can be applied to patients with a certain liver function reserve, either as a bridge treatment extending the waiting period for a liver transplant, or as a long-term, definitive therapy in case of satisfactory and stabilised liver function. As a rule however, the curative effect only comes after several days or weeks following intervention[69].

According to surveys published to date, it has been effective in ascites treatment in principle, and moreover, it can contribute to improvement of renal function[70,71].

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GUIDELINES FOR BASIC SCIENCE

Novel insight into mechanisms of cholestatic liver injury

Benjamin L Woolbright, Hartmut Jaeschke

Benjamin L Woolbright, Hartmut Jaeschke, Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160, United States
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Correspondence to: Hartmut Jaeschke, PhD, Professor, Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160, United States. hjaeschke@kumc.edu
Telephone: +1-913-5887969 Fax: +1-913-5887501
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Abstract

Cholestasis results in a buildup of bile acids in serum and in hepatocytes. Early studies into the mechanisms of cholestatic liver injury strongly implicated bile acid-induced apoptosis as the major cause of hepatocellular injury. Recent work has focused both on the role of bile acids in cell signaling as well as the role of sterile inflammation in the pathophysiology. Advances in modern analytical methodology have allowed for more accurate measuring of bile acid concentrations in serum, liver, and bile to very low levels of detection. Interestingly, toxic bile acid levels are seemingly far lower than previously hypothesized. The initial hypothesis has been based largely upon the exposure of μmol/L concentrations of toxic bile acids and bile salts to primary hepatocytes in cell culture, the possibility that *in vivo* bile acid concentrations may be far lower than the observed *in vitro* toxicity has far reaching implications in the mechanism of injury. This review will focus on both how different bile acids and different bile acid concentrations can affect hepatocytes during cholestasis, and additionally provide insight into how these data support recent hypotheses that cholestatic liver injury may not occur through direct bile acid-induced apoptosis, but may involve largely inflammatory cell-mediated liver cell necrosis.

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Key words: Bile acids; Cholestasis; Apoptosis; Necrosis; Neutrophils; Innate immunity; Bile duct ligation

Peer reviewers: Dr. Richard A Rippe, Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States; Bruno Stieger, Professor, Department of Medicine, Division of Clinical Pharmacology and Toxicology, University Hospital, 8091 Zurich, Switzerland

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CHOLESTATIC LIVER INJURY - CLINICAL RELEVANCE

A reduction in bile flow (cholestasis) can result from gall stones, impingement from a local tumor, intrahepatic cholestasis of pregnancy, genetic deficiency in bile export proteins or in autoimmune disorders amongst other etiologies[1,2]. Cholestasis results in a dramatic increase in liver and serum bile acid levels that eventually lead to acute liver toxicity, proliferation of bile ducts, and fibrosis progressing to cirrhosis[3]. Neonatal disorders such as progressive familial intrahepatic cholestasis and biliary atresia are particularly difficult to treat effectively[4]. While surgical means can prevent disease progression in the case of gall stones or biliary atresia, there are a limited number of treatment options pharmacologically for diseases such as primary sclerosing cholangitis or primary biliary cirrhosis[5]. Ursodiol, a pharmaceutical form of ursodeoxycholic acid, is the current mainline defense against cholestasis, although its efficacy is limited in

some circumstances[5,6].

While many of the etiologies of disease initiation are well described, the molecular mechanisms behind the early liver injury associated with cholestasis are extensively studied, but not well understood. A major hypothesis was based on the assumption that the buildup of bile acids in hepatocytes and blood results in hepatocellular apoptosis dependent upon bile acid concentrations[6,7]. While these studies on the direct toxicity of bile acids have been supported by many *in vitro* studies from multiple groups, recent methodology measuring the concentrations of individual bile acids after the onset of cholestasis *in vivo* has cast some doubt that bile acid levels may actually reach the levels necessary for cell toxicity[8-10]. A growing volume of work has instead focused on how sterile inflammation and innate immunity may result in the initial injury[11-15]. This review will discuss current and developing paradigms in the pathophysiology of early cholestatic liver injury, and provide insight into how recent advances in methodology may affect these theories.

DIRECT BILE ACID TOXICITY AS A MODEL FOR CHOLESTATIC LIVER INJURY

High concentrations of bile acids given to cultured hepatocytes *in vitro*[16] and bile acid-induced cholestasis[17], or bile duct ligation *in vivo*[18], have long been established as models of toxicity to hepatocytes. After the discovery that ursodeoxycholic acid could be used pharmacologically as a choleretic agent with protective effects against cholestasis[19], a renewed interest in the subject emerged. While previous papers had focused on the detergent properties of amphipathic bile acids[20,21] recent research resulted in a series of papers illustrating a detailed mechanism of how toxic bile acids or the respective bile salt, could induce apoptosis in primary human and rat hepatocytes[22-27]. Much of this work has spearheaded the current revival of direct bile acid toxicity as a model of cholestasis, or liver injury, *in vitro* and *in vivo*.

Apoptosis and intracellular signaling mechanisms in bile acid-induced toxicity

The most commonly studied bile acid for its toxicity to hepatocytes is glycochenodeoxycholic acid (GCDCA), or its bile salt glycochenodeoxycholate, due to it being a major constituent of human serum and bile after cholestasis[28] and its relatively higher level of toxicity when compared to other bile acids[29]. Galle *et al*[22] first established GCDCA as toxic to primary human hepatocytes and in the same paper also established ursodeoxycholate (UDCA) as protective against the GCDCA-induced injury. Due to the limited availability of primary human hepatocytes, much of the subsequent research into mechanism of injury occurred in rodent, especially rat hepatocytes. GCDCA-induced hepatocellular death was first established as apoptotic using electron microscopy[23]. Incubation of primary rat hepatocytes with 50 μmol/L GCDCA induced DNA fragmentation, chromatin margination and condensation, cellular shrinkage and blebbing of the membrane with intact organelles enclosed, all of these classical hallmark characteristics of apoptosis[23]. While it has been extensively shown that DNA fragmentation is common to both necrosis and apoptosis[30-33], the morphological changes present after GCDCA exposure provided strong evidence of bile acid-induced apoptosis of primary hepatocytes. In addition, caspase activation is a feature of GCDCA-induced apoptosis[27,34-36] and caspase inhibitors were shown to protect[27,34,36].

Investigation into intracellular mechanisms of bile acid toxicity showed cellular apoptosis was associated with mitochondrial instability and mitochondrial membrane permeability transition pore (MPTP) formation that was preventable by either UDCA, ketohexoses such as fructose, or cyclosporine A and trifluoroperazine[36-39]. As mitochondria are essential for ATP production and GCDCA depleted ATP levels[29], the idea that UDCA, or other protective interventions, were sustaining ATP levels was tested; however, protection was not due to reduced ATP depletion, but by blockage of the MPTP[37]. This suggested that UDCA may prevent injury during cholestasis not only by enhancing bile flow, but by preventing initiation of the MPTP[37], a facet which has been confirmed in other models[40-42]. Mitochondrial reactive oxygen species (ROS) production and MPTP initiation was later also shown in human hepatocytes exposed to toxic bile acids such as GCDCA indicating the mitochondrial dysfunctions occur in more than just rodent species[43]. Adult rats fed a diet supplemented with deoxycholic acid (DCA) for 10 d also showed significant mitochondrial swelling, peroxynitrite formation and ROS production[44]. These data suggest that exposure of cultured hepatocytes to high levels of hydrophobic bile acids causes formation of ROS, reactive nitrogen species, and initiation of the MPTP, which is directly involved in the injury. In immortalized cell lines transfected with the basolateral transporter NTCP, bile acids like GCDCA could directly stimulate both Fas-dependent apoptosis *via* activation of Fas receptor[26] and Fas-independent apoptosis *via* previously established mitochondrial damaging mechanisms[41,42]. These experiments indicate that uptake of bile acids can cause both intrinsic and extrinsic apoptosis, and that cell death could be ameliorated by protecting against MPTP formation. These observations were critical in establishing the role of intracellular signaling and mitochondrial stress in the pathophysiology of bile acid-induced toxicity, as previous hypotheses revolved largely around the idea of inherent bile acid toxicity.

Although GCDCA and its bile salt have been extensively studied, other toxic bile acids have also effects on intracellular signaling mechanisms. Taurolithocholic acid (TLCA), taurochenodeoxycholic acid, and DCA increase intracellular ROS species in hepatocytes[45,46] and toxicity from these bile acids is responsive to antioxidants such as N-acetylcysteine and α-tocopherol[45]. DCA has also been shown to induce apoptosis and mitochondrial

instability in adult rats and primary hepatocytes at high concentrations[44,47,48]. Interestingly the taurine conjugate of UDCA, TUDCA, is protective against bile acid-induced apoptosis through activation of mitogen-activated protein kinase survival pathway signaling including p38 and extracellular signal-regulated kinase[55]. While GCDCA may be one of the most toxic and perhaps the most interesting bile acid scientifically and mechanistically, the total bile acid milieu probably contributes to the overall effect more than a single bile acid, with anti-apoptotic bile acids such as TUDCA and UDCA counteracting some of the effects of pro-apoptotic bile acids such as GCDCA.

Bile acids are also known to target intracellular nuclear receptors, especially the liver X receptor (LXR) family such as farnesoid X receptor (FXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR) and more[49]. The first LXRs identified as bile acid receptors were FXR[50,51], LXR[52], and later PXR[53]. FXR has been established as a key regulator in bile acid synthesis and transport[54]. Agonism of FXR with the synthetic drug GW4064 is hepatoprotective against both intra- and extrahepatic cholestasis[55] and agonism of PXR is protective against certain bile acid-induced cholestatic syndromes[56]. Nevertheless, the FXR knockout mouse were also protected against bile duct ligation-induced injury[57,58], which was found to be *via* increases in phase I detoxifying reactions and renal excretion of bile acids[59]. This suggests that both agonism and antagonism may be beneficial, depending on the type of cholestasis and the individual response to alterations in master regulators such as FXR. There is evidence for reduced bile acid load by either a reduction in synthesis in the case of agonism or enhanced excretion in antagonism of FXR. In cases of agonism a reduced bile acid load also correlated with a reduction in the hepatic inflammatory response in both steatotic and non-steatotic models[55,60] and loss of FXR has also protected against inflammatory injury in other organs[61]. These data indicate that increasing bile acid levels may have a strong signaling effect through FXR, PXR and more in hepatocytes that results in decreased bile acid synthesis, increased bile acid excretion and recruitment of inflammatory cells to the area. Early growth response factor-1 (Egr-1) has been clearly established as a master regulator in the sterile inflammatory response that hepatocytes express after exposure to high levels of bile acids[62]. Future research investigating interplay between FXR and Egr-1 in this signaling axis may be fruitful.

Bile acid concentrations: The in vivo to in vitro shift and back again

While it seems undeniable that bile acids have the capacity to elicit multiple different signaling cascades ranging from mitochondrial dysfunction and cell death to bile acid synthesis and glucose homeostasis[49,63], there remains some debate over the models used to dissect these actions and their physiological relevancy. In particular, there appears to be a discrepancy between the concentration of bile acids used *in vitro* to cause toxicity, and the levels of bile acids occurring during cholestasis both in rodents and in man[8,9].

Table 1 Human plasma bile acid concentrations in healthy volunteers and patients with extrahepatic cholestasis

BA	Healthy volunteers (nmol/L)	Cholestatic patients (nmol/L)
CDCA	257 ± 56	84 ± 60
TCDCA	120 ± 22	16 578 ± 4370
GCDCA	776 ± 112	28 535 ± 5734
CA	182 ± 83	173 ± 137
TCA	180 ± 47	54 487 ± 13 419
GCA	233 ± 56	56 222 ± 15 714
UDCA	138 ± 25	20 ± 11
TUDCA	5 ± 1	75 ± 22
LCA	13 ± 2	2 ± 1
TLCA	23 ± 4	12 ± 2
GLCA	16 ± 4	11 ± 2
LCA-S	7 ± 1	4 ± 2
DCA	387 ± 66	21 ± 16
TDCA	45 ± 12	213 ± 71
GDCA	246 ± 43	418 ± 91

BA: Bile acid; CA: Cholic acid; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; GCA: Glycocholic acid; GCDCA: Glycochenodeoxycholic acid; GDCA: Glycodeoxycholic acid; LCA: Lithocholic acid; TCA: Taurocholic acid; TCDCA: Taurochenodeoxycholic acid; TLCA: Taurolithocholic acid; TUDCA: Tauroursodeoxycholic acid; UDCA: Ursodeoxycholic acid; LCA-S: Lithocholic acid sulfate; GLCA: Glycolithocholic acid; TDCA: Taurodeoxycholic acid. Data adapted from Trottier *et al*[9].

Table 1 illustrates the increases in serum bile acids found in sex and age matched patients after multiple forms of cholestasis in man[9]. Full details on the patients are provided by Trottier *et al*[9] and Lai *et al*[64]. In brief, bile acid concentrations were measured in human serum from healthy volunteers and from patients with extrahepatic cholestasis[9]. Seventeen patients (8 men and 9 women) were recruited to the study. Clinical diagnoses of the patients included 8 common bile duct stones, 5 pancreatic tumors, 1 bile duct tumor, 1 benign common bile duct stenosis and 1 chronic pancreatitis. Age and sex matched (20 male, 20 female) non-cholestatic volunteers were used as controls[9], selected from the Genetics of Lipid Lowering Drugs and Diet Network study[64].

Previous work suggests serum bile acid levels are highly similar to total liver levels when comparing bile acid concentrations *in vivo*[8]. The bile acids that show the greatest increase in concentration in the serum are not toxic bile acids, but largely non-toxic bile acids such as TCA and GCA in man[9] or TCA, α- and β-muricholic acid (MCA) and TMCA in mice[8]. Levels of the primary toxic bile acid in humans, GCDCA, rise substantially, to almost 30 μmol/L under severe cholestatic conditions as measured by serum levels in man[9]. However, studies with primary human hepatocytes have shown little to no increase in toxicity until levels greater than 100 μmol/L for 24 h with significant toxicity beginning around 500 μmol/L[22,65]. The only paper to demonstrate toxicity at a level close to 30 μmol/L reported that GCDCA showed morphological characteristics in primary human hepatocytes that resembled

Table 2 Murine plasma bile acid concentrations after bile duct ligation

BA	Sham control (μmol/L)	BDL 6 h (μmol/L)
TMCA	0.229 ± 0.029	1.003 ± 133
TCA	0.122 ± 0.027	1.072 ± 137
TCDCA	0.029 ± 0.003	7 ± 0.557
GCA	0.007 ± 0.0002	1 ± 0.118
GCDCA	0.003 ± 0.003	0 ± 0
αMCA	0.697 ± 0.273	87 ± 41
βMCA	0.175 ± 0.23	253 ± 100
CA	0.219 ± 0.059	17 ± 10
CDCA	0.0001 ± 0.0001	0.144 ± 0.085
TUDCA	0.031 ± 0.002	16 ± 14
TDCA	0.016 ± 0.004	0.330 ± 0.107
TLCA	0.007 ± 0.002	0.027 ± 0.002
UDCA	0.131 ± 0.002	0.492 ± 0.215
DCA	0.042 ± 0.018	0.109 ± 0.032
LCA	0.054 ± 0.013	0.033 ± 0.022

BA: Bile acid; CA: Cholic acid; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; GCA: Glycocholic acid; GCDCA: Glycochenodeoxycholic acid; GDCA: Glycodeoxycholic acid; LCA: Lithocholic acid; TCA: Taurocholic acid; TCDCA: Taurochenodeoxycholic acid; TLCA: Taurolithocholic acid; TUDCA: Tauroursodeoxycholic acid; UDCA: Ursodeoxycholic acid; LCA-S: Lithocholic acid sulfate; GLCA: Glycolithocholic acid; TDCA: Taurodeoxycholic acid; BDL: Bile duct ligation. Data adapted from Zhang *et al*[8].

apoptosis and a 60% increase in DNA fragmentation at 50 μmol/L[66]. However, a significant increase in serum aspartate aminotransferase activities over control levels was only observed at 100 μmol/L GCDCA[66]. These data suggest that direct bile acid toxicity may not be the main mechanism of cholestatic liver injury in humans and seem to be supported by findings in mouse models of obstructive cholestasis[8,15]. Table 2 shows plasma bile acid concentrations in sham-operated controls or after 6 h of bile duct ligation (BDL)[8]. This is a time point corresponding with either maximal or near maximal increases in bile acid concentrations. Data are expressed as mean ± SE of $n = 6$ per group. For toxic bile acids such as GCDCA or LCA, the levels required to initiate toxicity in murine hepatocyte cultures are up to 3000-fold higher than the corresponding bile acid levels *in vivo*[8]. In the case of GCDCA, the most commonly used bile acid for *in vitro* toxicity, *in vivo* levels never exceed about 24 nmol/L throughout the first 3 d of injury[8]. This compares to 50 μmol/L GCDCA that is required to cause toxicity in cultured rodent hepatocytes *in vitro*[23,26]. In addition, during cholestasis *in vivo*, hepatocytes are exposed to a mixture of bile acids, some of which have actually an anti-apoptotic effect by causing the upregulation of inhibitor of apoptosis proteins, which may serve to counterbalance the apoptotic effect of specific bile acids[67,68].

While it is known that cultured hepatocytes lose expression of bile acid transporters such as NTCP and the OATPs over time in culture, cells freshly taken from the liver express nearly the same amount of transporter as whole liver tissue[69], and nearly the same level at 3 h after plating[70], when many of these experiments begin[23,71,72]. Even given a 30% decrease in mRNA levels of transporters such as NTCP 3 h after plating, cells maintain over 50% of their NTCP function up to 24 h after plating as measured by kinetic parameters[70]. Thus, it stands to reason that these hepatocytes are concentrating bile acids *in vitro* at levels within a 2 fold difference of the *in vivo* physiology, yet it takes multiple orders of magnitude, up to a 3000 fold difference in the case of GCDCA, to account for the difference between bile acid levels used on rodent hepatocytes *in vitro* and levels observed in murine samples *in vivo*. Still, even given that serum or liver levels may underestimate the true concentrations of bile acids hepatocytes are exposed to after BDL when bile leaks back into the parenchyma[73,74], the principle bile acid composition in bile is similar to serum, i.e. the non-toxic bile acids predominate, thus it is unlikely that cells are exposed to micromolar concentrations of the *toxic* bile acids *in vivo*[75].

INFLAMMATORY LIVER INJURY AFTER BDL - A NOVEL HYPOTHESIS

If bile acids are not directly responsible for cholestatic liver injury *in vivo,* then what is the mechanism of injury, and what role do bile acids play in the pathophysiology and progression of the injury? Accumulation of neutrophils, largely around sites of cell injury, within 24 h after bile duct ligation has been reported numerous times[11,12,76,77]. In addition, an increase in macrophage accumulation is observed approximately one week post ligation[77,78]. Figure 1 shows typical histology and neutrophil extravasation after bile duct ligation in mice. H&E staining shows multiple areas of hepatic focal necrosis throughout the liver. Neutrophils are present around the site of injury as early as six hours post BDL, and peak around 48-72 h after BDL, which is also the peak of liver injury[8,11,77].

Neutrophil accumulation has been directly associated with the injury, as deficiency of adhesion molecules such as ICAM-1 or CD18 reduced the number of neutrophils that extravasate into liver parenchyma, and drastically reduced liver injury[11,12]. A neutrophil is a particularly toxic leukocyte due to its capacity to produce superoxide *via* nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the potent oxidant hypochlorous acid *via* myeloperoxidase[79]. Neutrophil accumulation after bile duct ligation has been associated with an increase in myeloperoxidase activity[80] and intracellular chlorotyrosine adduct formation in hepatocytes[11,12,81]. This indicates that neutrophil-derived hypochlorous acid can cause an intracellular oxidant stress in hepatocytes, which is responsible for cell injury[82,83]. Inhibition of NADPH oxidase reduces neutrophil-mediated oxidant stress and protects against neutrophil cytotoxicity[84,85]. In addition, hepatic NADPH oxidases located in hepatic stellate cells (Nox1) and Kupffer cells (Nox2) contribute to BDL-induced fibrosis[86,87] but their contribution to the initial liver injury (bile infarcts) has not been determined.

Together these data strongly implicate neutrophils

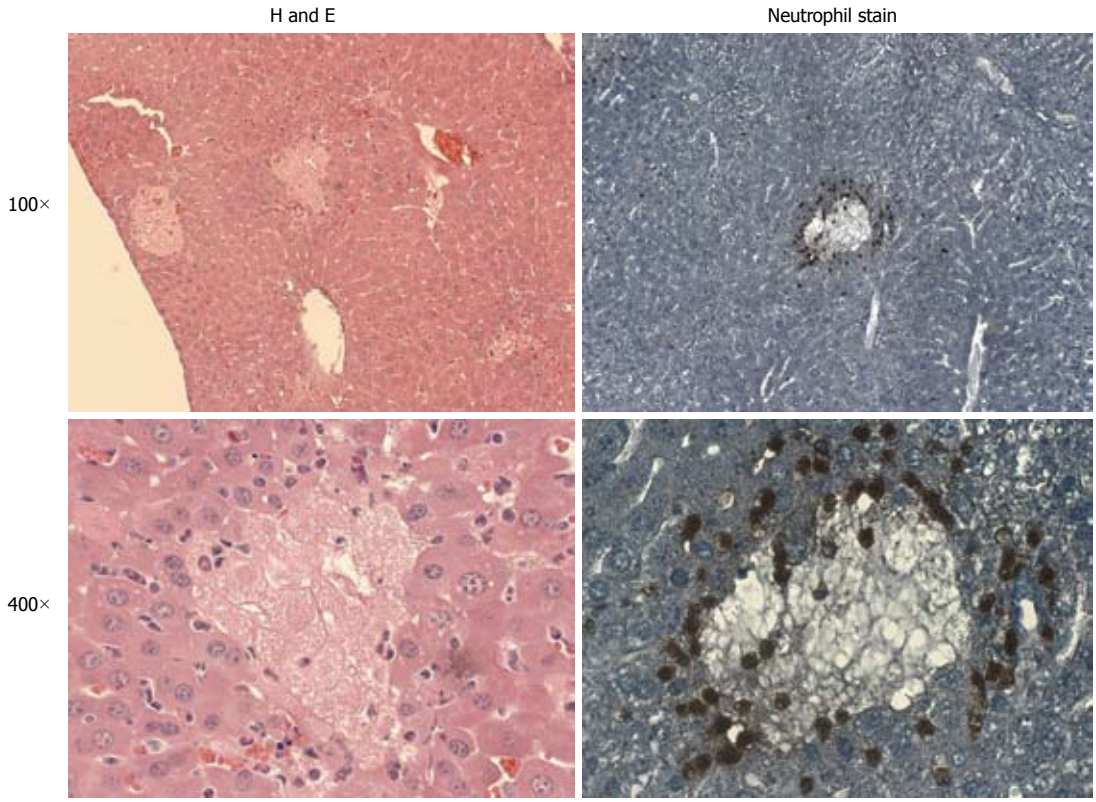


Figure 1 Sample liver sections from C57Bl/6 mice illustrating neutrophil accumulation after bile duct ligation. C57BL/6 mice were subjected to bile duct ligation and sacrificed 24 h later. Hematoxylin and eosin (H and E) stained sections showing focal necrosis at 100× and 400× magnification. Sections stained with an anti-neutrophil antibody. Neutrophils are present largely around areas of focal necrosis in this model.

in the pathogenesis of early cholestatic liver injury. In support of this hypothesis is recent data that exposure of cultured hepatocytes to both toxic and non-toxic bile acids at levels that recapitulate the *in vivo* concentrations result in dramatic increases in chemotactic factors such as MIP-2 and mKC, and adhesion molecules such as ICAM-1, all factors that can be involved in hepatic neutrophil recruitment[15]. The inflammatory mediator induction is dependent on the activation of the transcription factor Egr-1 in bile duct-ligated mice and in bile acid-exposed hepatocytes[15,62]. Egr-1 expression is also increased in human patients with cholestatic liver disease and the hepatic Egr-1 levels correlate with interleukin-8 and ICAM-1 induction[15]. Therefore, we initially hypothesized that after BDL, bile leaks back into the parenchyma after rupture of bile ducts and hepatocytes are locally exposed to high concentrations of pro-inflammatory bile acids such as TCA, β-MC and TMC that initiate an inflammatory response[8,13,15]. Additionally these hepatocytes are exposed to low concentrations of cytotoxic bile acids such as DCA, TLCA, and GCDCA that may initiate a low level of mitochondrial dysfunction that stresses the hepatocytes, but is insufficient to directly kill the cells[8,29,37,38,41-43]. This bile acid-induced stress triggers the expression of ICAM-1, MIP-2, and mKC in hepatocytes[8,15]. The subsequent release of these CXC chemokines can activate neutrophils in circulation[88] and provide a chemotactic gradient for the extravasation of neutrophils, which then attack the stressed hepatocytes and cause cell death through reactive oxygen formation[11,12,81,82]. Given a dramatic enough increase in toxic bile acid concentrations by feeding e.g. a lithocholic acid-containing diet[89], it is possible to directly cause cell death *via* bile acid toxicity but independent of neutrophils (Woolbright and Jaeschke unpublished observation). However the *in vivo* bile acid concentrations in models such as BDL are insufficient to meet this threshold, and thus the neutrophil-mediated cell death is dominant.

APOPTOTIC AND NECROTIC CELL DEATH AFTER BDL

A central but controversial issue in the debate on the mechanisms of cell death in cholestatic liver disease is whether the injury occurs through apoptosis or necrosis[90]. This is a key point as the therapeutic options to prevent injury will progress in two very different directions depending on the answer. Necrosis is characterized by cellular swelling, membrane blebbing, DNA fragmentation and release of cellular components, whereas apoptosis is characterized by cellular shrinking, caspase activation, DNA fragmentation, chromatin condensation with initially no release of cellular contents[32]. These processes lead to dramatically different outcomes after cell death in regards to progression of disease with ne-

crosis leading to increased inflammation and further aggravation by inflammatory cells[81,90,91].

Although there is no question that high concentrations of certain hydrophobic bile acids can cause apoptotic cell death in cultured rodent hepatocytes[22-27, 34-42,67,68] as discussed earlier, the pathophysiological relevance of these observations for *in vivo* models, e.g., BDL, is questioned due to the fact that the pro-apoptotic bile acids do not achieve sufficiently high levels *in vivo* to directly cause cell death[8,9]. Thus, the evaluation of the mechanism of cell injury has to be based on the *in vivo* evidence. Early studies concluded that BDL-induced liver injury was caused by apoptosis mainly due to the positive terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay[92-94] which is clearly not specific for apoptotic cell death[30-32]. In addition, immunofluorescence was used to detect cells with active caspases 3/7[95] but this was not confirmed by others[96]. Additional arguments for apoptosis were the reduced BDL-induced liver injury in Fas receptor-deficient *lpr* mice[94] and after anti-sense treatment to reduce Bid expression[97]. However, *lpr* mice are protected due to the reduced inflammatory response[98] and genetically Bid-deficient mice did not show reduced liver injury[96]. In contrast, numerous studies did not find any morphological evidence of apoptosis[11,12,68,98-101] and no evidence of caspase activation (enzyme activity and/or active fragments) or immunohistochemical staining for cytokeratin 18 cleavage products or active caspase-3/7[96,98,99]. In addition, potent caspase inhibitors, which effectively eliminated caspase activation and cell death in Fas- or TNF-mediated apoptosis models[98,102], did not protect against BDL-induced liver injury[98]. The one study reporting reduced injury after a caspase inhibitor in the absence of relevant caspase activation[95] may be due to off-target effects on other proteases[103]. Likewise, the anti-sense knock-down of Bid gene expression may have caused compensatory protective gene activation, which may have attenuated BDL-induced liver injury[95]. Clearly, Bid-deficient mice were not protected against BDL-induced liver injury[96]. The cathepsin B knockout mouse also showed protection against apoptotic injury[92], although apoptosis was assessed entirely based on the TUNEL assay. While the reduction in injury was attributed to lack of cathepsin B, and thus a lack of apoptotic processing proteins, the paper also showed a significant reduction in the neutrophilic inflammatory response[92]. Thus, the reduced injury in cathepsin B-deficient mice may have been due to reduced inflammation rather than apoptotic cell death. Taken together, the preponderance of experimental evidence supports the conclusion that liver injury after BDL involves mainly necrotic cell death.

In conclusion, when using morphological criteria of apoptosis and caspase activation in combination with positive controls of apoptotic cell death *in vivo*, the overwhelming experimental evidence supports the conclusion that there is no relevant apoptotic cell death but almost exclusively necrosis after BDL. The main paradigm shift comes from the recognition that the well-known hepatotoxic bile acids never reach concentrations in the liver after BDL that would directly cause cell death. In contrast, the most common bile acids that reach close to mmol/L levels trigger inflammatory mediator formation, which initiates an inflammatory response and cell death caused by neutrophils through oxidant stress. Initial data from human patients with cholestatic liver disease appear to confirm several aspects of the mouse model. Thus, the focus of future research should be on evaluating the cell stress mechanisms in hepatocytes considering pathophysiologically relevant bile acid mixtures and on further characterizing the initiation of the inflammatory response after BDL. Understanding these early injury mechanisms may not only reveal therapeutic approaches to reduce liver injury but also the subsequent fibrosis.

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Consensus statement AIGO/SICCR diagnosis and treatment of chronic constipation and obstructed defecation (Part Ⅱ: Treatment)

Antonio Bove, Massimo Bellini, Edda Battaglia, Renato Bocchini, Dario Gambaccini, Vincenzo Bove, Filippo Pucciani, Donato Francesco Altomare, Giuseppe Dodi, Guido Sciaudone, Ezio Falletto, Vittorio Piloni

Antonio Bove, Vincenzo Bove, Gastroenterology and Endoscopy Unit, Department of Gastroenterology, AORN "A. Cardarelli", 80131 Naples, Italy
Massimo Bellini, Dario Gambaccini, Gastrointestinal Unit, Department of Gastroenterology, University of Pisa, 56100 Pisa, Italy
Edda Battaglia, Gastroenterology and Endoscopy Unit, Cardinal Massaja Hospital, 14100 Asti, Italy
Renato Bocchini, Gastroenterology Unit, M. Bufalini Hospital, 47023 Cesena, Italy
Filippo Pucciani, Department of Medical and Surgical Critical Care, University of Florence, 50141 Florence, Italy
Donato Francesco Altomare, Department of Emergency and Organ Transplantation, University of Bari, 70124 Bari, Italy
Giuseppe Dodi, Department of Oncological and Surgical Sciences, University of Padua, 35126 Padua, Italy
Guido Sciaudone, Division of General and Geriatric Surgery, Second University of Naples, 80131 Naples, Italy
Ezio Falletto, Sixth Division of University Surgery, Department of General Surgery, San Giovanni Battista Hospital, 10126 Turin, Italy
Vittorio Piloni, Diagnostic Imaging Centre "N. Aliotta", Villa Silvia - Senigallia, I-60100 Ancona, Italy
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Correspondence to: Antonio Bove, MD, Gastroenterology and Endoscopy Unit, Department of Gastroenterology, AORN "A. Cardarelli", Via Cardarelli, 9, 80131 Naples, Italy. 3392982380@fastwebnet.it
Telephone: +39-81-7474034 Fax: +39-81-7474034
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Abstract

The second part of the Consensus Statement of the Italian Association of Hospital Gastroenterologists and Italian Society of Colo-Rectal Surgery reports on the treatment of chronic constipation and obstructed defecation. There is no evidence that increasing fluid intake and physical activity can relieve the symptoms of chronic constipation. Patients with normal-transit constipation should increase their fibre intake through their diet or with commercial fibre. Osmotic laxatives may be effective in patients who do not respond to fibre supplements. Stimulant laxatives should be reserved for patients who do not respond to osmotic laxatives. Controlled trials have shown that serotoninergic enterokinetic agents, such as prucalopride, and prosecretory agents, such as lubiprostone, are effective in the treatment of patients with chronic constipation. Surgery is sometimes necessary. Total colectomy with ileorectostomy may be considered in patients with slow-transit constipation and inertia coli who are resistant to medical therapy and who do not have defecatory disorders, generalised motility disorders or psychological disorders. Randomised controlled trials have established the efficacy of rehabilitative treatment in dys-synergic defecation. Many surgical procedures may be used to treat obstructed defecation in patients with acquired anatomical defects, but none is considered to be the gold standard. Surgery should be reserved for selected patients with an impaired quality of life. Obstructed defecation is often associated with pelvic organ prolapse. Surgery with the placement of prostheses is replacing fascial surgery in the treatment of pelvic organ prolapse, but the efficacy and safety of such procedures have not yet been established.

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Peer reviewer: Timothy R Koch, MD, FACG, Professor of Medicine, Gastroenterology, Washington Hospital Center, Georgetown University School of Medicine, POB North, Suite 3400, 106 Irving Street, NW, Washington, DC 20010, United States

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MEDICAL AND REHABILITATIVE TREATMENT

Behavioural modification is considered to be the first-line treatment in patients with symptomatic chronic constipation. If the behavioural modification proves unsuccessful, various pharmacological approaches are available.

Behavioural modification

Recommendations for lifestyle changes in patients with chronic constipation are based on the widespread belief that constipation is associated with low physical activity, reluctance to defecate whenever the need is felt, and poor fluid intake.

Can behavioural changes help the patient with chronic constipation?

Physical exercise: Epidemiological studies report that constipation is more frequent in subjects with a sedentary lifestyle[1]. Physical activity can increase colonic transit time[2] and reduce other symptoms of constipation in elderly subjects[3]. Trials evaluating the effect of exercise in constipated patients are lacking. Increased physical activity is often recommended for patients with chronic constipation, but there is no evidence that constipation can be improved by increased physical activity.

Defecation habits: Patients with chronic constipation are often instructed to defecate when the need is felt and to try to defecate at the same time every day, ideally upon awakening and after meals, when the colonic motor activity is highest. This recommendation is based on the observation that many people with normal colonic activity routinely defecate at the same time each day[4]. Trials evaluating this recommendation in constipated patients are also lacking.

Increased fluid intake: It is generally believed that increased fluid intake improves constipation. In one trial, healthy volunteers were given increasing amounts of liquids, up to 2 L/d. The volume of urine increased, but the stool frequency did not[5]. This finding is not surprising because the absorption capacity of the small intestine is 7-10 L/d. Trials evaluating the effect of increased liquid intake in constipated patients are lacking, and there is no evidence that constipation can be improved by increasing oral fluid intake, unless the patient is dehydrated[6]. Suggestions for behavioural changes are not usually helpful. These recommendations are supported by Level V evidence, Grade C recommendation.

PHARMACOLOGICAL THERAPY

Various drugs are available to treat chronic constipation (Table 1). Laxatives generally can resolve the symptoms of constipation, but few rigorous studies on their effectiveness have been conducted[7]. Placebo-controlled trials conducted over a sufficient period of time are needed to demonstrate the actual efficacy of an agent. Many trials have been of short duration (4 wk), which limits the validity of their conclusions because cognitive studies have shown that half of all patients become dissatisfied with their therapy over time[8]. Furthermore, the results of different studies are not always comparable; the definition of constipation may not be sufficiently specific, and the therapeutic end-point may simply be stool frequency, without taking other symptoms into account.

The resolution of constipation-related symptoms is an important therapeutic target because constipation is a complex condition, with hard stool, straining, incomplete evacuation, bloating, and abdominal discomfort. Infrequent bowel movements are not always present, and infrequent bowel movements are certainly not the most unpleasant[8] symptom of constipation. Additionally, investigators have only recently begun to address the important problem of quality of life in patients with constipation, which has been shown to worsen as constipation-related symptoms increase[9-11].

Defining constipation remains difficult[12]. Patients and physicians often have different feelings and opinions on the matter; patients use the word "constipation" to mean the annoying symptoms related to defecation, but physicians use this term to describe infrequent bowel movements[13,14]. Constipation has two different but overlapping pathophysiological characteristics: delayed transit and evacuation disorders. The Roma Ⅲ criteria[15,16] were developed by an international panel of experts and have been applied in several clinical trials of laxatives. These criteria have also been adopted in this consensus statement and are useful in clinical practice and for clinical research.

Bulking laxatives

Bulking laxatives consist of fibre. These agents must be ingested with sufficient amounts of water to increase the weight of the faeces. Their action begins within 12 to 72 h, but their effectiveness should be assessed after a period of some weeks. There are two types of fibre: in-

Table 1 Classes of drugs used to treat chronic constipation

Type of medication	Drugs
Laxatives	
Bulking (insoluble and soluble fibres)	Bran, methylcellulose, psyllium
Osmotic	Lactulose, sorbitol, magnesium hydroxide, magnesium salts, polyethylene glycol
Stimulant	Anthraquinone derivatives: senna, aloe, cascara
	Diphenylmethane derivatives: bisacodyl, sodium picosulfate
Softening	Liquid paraffin (vaseline oil), docusate, glycerine
Serotoninergic enterokinetic agents	Tegaserod, prucalopride, renzapride
Prosecretory agents	Lubiprostone, linaclotide
Gastrointestinal μ-opioid antagonists	Methylnaltrexone, alvimopan
Probiotics	Bifidobacterium, lactobacillus
Colchicine	

soluble and soluble. Insoluble fibre consists of bran containing cellulose, hemicellulose and lignin. Galattomannan, pectin, gum and mucilage are types of soluble fibre that can be found in fruits and in some vegetables. Most types of soluble fibre are completely fermented in the colon, except psyllium, which is only partially fermented. Fermentation increases the production of short-chain fats and gas; therefore, one side effect of bulking laxatives is bloating.

What evidence is there for the effectiveness of added fibre intake?

Current guidelines recommend the use of fibre in both dietary and supplement form for the first-line treatment of chronic constipation[17], but a recent review showed that there is little evidence to support this approach[18].

Trials of insoluble fibre

There have been only two well-conducted placebo-controlled trials of insoluble fibre[18]; the first used bran and the second used rye bread. The first trial was a crossover study that enrolled 24 patients. In this study, the effectiveness of bran was documented only if the placebo was given before the bran[19]. The second trial studied 29 patients and compared a diet rich in rye bread to a diet containing low-fibre bread. The bowel movement frequency and difficulty in defecation significantly improved with a diet rich in rye bread[20].

In a randomised trial, 117 constipated patients were treated with bran plus water: one group was told to drink water as desired, whereas the other group was instructed to consume 2 L of water per day. The ingestion of bran plus 2 L of water increased the stool frequency ($P < 0.001$) and reduced the use of rescue laxatives[21]. Fibre supplementation may lead to the increased use of enemas and suppositories[22]. The data regarding insoluble fibre are conflicting[18]. Treating constipation with bran is supported by Level Ⅲ evidence, Grade C recommendation.

Trial of soluble fibre

Placebo-controlled trials of psyllium: Psyllium fibre is partially soluble and is the most studied type of fibre. Three placebo-controlled trials on the efficacy of psyllium have been published[23-25]. Two found that psyllium was superior to a placebo in increasing the frequency of defecation ($P < 0.05$) and improving the consistency of the stool ($P < 0.05$)[23,24]. Ashraf *et al*[23] conducted a well-designed study, but it only lasted 8 wk and only enrolled 22 patients. The third study reported no significant difference between psyllium and a placebo[25].

Studies comparing psyllium with other laxatives: One trial reported no difference in the stool frequency between a regimen of senna plus psyllium and psyllium alone[26]. A study comparing psyllium and laxatives (lactulose, bisacodyl, docusate, senna and magnesium salts) found that psyllium was more effective in increasing the stool frequency and improving the stool's consistency[27]. The use of psyllium is supported by Level Ⅱ evidence, Grade B recommendation.

Osmotic laxatives

Osmotic laxatives attract water into the colon by osmosis. Sugar-based laxatives and polyethylene glycol (PEG) are effective after 24 to 48 h. Magnesium hydroxide and magnesium salts are effective after 6-8 h.

How effective are osmotic laxatives?

Placebo-controlled trials of lactulose: Three trials have shown the effectiveness of lactulose[28-30] in increasing the stool frequency ($P < 0.05$). These trials may be biased, however, because of the number of patients enrolled and their age; furthermore, the sex distribution and treatment duration were not specified. The side effects of lactulose include bloating, nausea and abdominal cramps.

Trials of lactulose *vs* other laxatives: Several trials have compared lactulose with other laxatives. Lactulose was shown to be less effective than PEG[31,32]. Psyllium plus senna was shown to be more effective than lactulose, but this combination can cause incontinence[33-35]. No difference was found between lactulose and psyllium[36] or lactulose and sorbitol[37], but nausea was reported more frequently in patients treated with lactulose ($P < 0.05$)[37]. The recommendation regarding sorbitol could not be graded because of insufficient data; the trial only enrolled men, and the randomisation procedure was not described[37]. The use of lactulose is supported by Level Ⅱ evidence, Grade B recommendation.

Magnesium hydroxide and magnesium salts: There is only one study in the literature that compares magnesium hydroxide to bulking laxatives[38]. Published in 1987 and focusing on elderly patients, this study has various drawbacks. However, in patients receiving magnesium hydroxide, the stool frequency was higher ($P < 0.001$), and the use of rescue laxatives was lower ($P < 0.01$).

The possible risk of hypermagnesemia with these agents must be mentioned[38]. Hypermagnesemia was not reported in this study but can occur in patients suffering from renal disease[39].

No clinical trials using magnesium salts (Epsom salts, English salt) have been published in the last 40 years. The use of magnesium hydroxide is supported by Level V evidence, Grade C recommendation.

Placebo-controlled trial of PEG: PEG is an organic polymer that is not degraded by the intestinal flora. The effectiveness of PEG has been documented in numerous trials[40-44]. PEG increased the stool frequency ($P < 0.01$) while improving the stool consistency[40,41,43] and reducing other symptoms of constipation[41,43]. Iso-osmotic or hypo-osmotic solutions of PEG consistently improved the frequency of bowel movements compared with the frequency before treatment ($P < 0.001$)[45]. PEG was well tolerated, and side effects (abdominal cramps, flatulence, nausea) were rare.

Trials of PEG *vs* other laxatives: PEG is more effective than lactulose[31,32] in increasing the stool frequency and improving the stool's consistency. In patients treated with PEG, there are also lower rates of rescue medication use and flatulence. One trial showed that PEG was more effective than Tegaserod[46]. PEG is a pillar in the treatment of chronic idiopathic constipation because of its high efficacy. There is evidence that PEG provides significant benefits compared with placebos and other laxatives. Furthermore, retrospective studies show that PEG remains effective for up two years of treatment[46,47]. The use of PEG is supported by Level I evidence, Grade A recommendation.

Stimulant laxatives

Stimulant laxatives are not absorbed and have a prokinetic effect in the colon; they stimulate the production of secretions and reduce the absorption of water and electrolytes. Stimulant laxatives begin to take effect 6 to 12 h after administration. Bisacodyl and sodium picosulfate (SPS) are prodrugs[48].

How effective are the stimulant laxatives?

Placebo-controlled trial of stimulant laxatives: Although stimulant laxatives have been used for many years to treat patients with constipation and are often used as rescue medications in clinical trials of other laxatives, only recently have placebo-controlled trials of stimulant laxatives been conducted. SPS[49] and oral bisacodyl[50] increase the stool frequency ($P < 0.0001$) while improving the stool consistency and decreasing the symptoms of constipation and the use of rescue medications ($P < 0.01$). These drugs are well tolerated and appear to generally improve the patient's quality of life.

Comparison of stimulant laxatives and other laxatives

Senna plus a bulk laxative[33,34] is more effective than lactulose ($P < 0.05$) but less effective than psyllium alone[26]. The faeces are softer with lactulose than with stimulant laxatives ($P < 0.001$)[51].

Adverse effects of stimulant laxatives include abdominal cramps and diarrhoea. Experimental studies have shown that stimulant laxatives do not damage the colonic epithelium[52]. Hepatotoxicity has been reported with some products; anthraquinone derivatives can cause melanosis of the colon.

Some physicians fear that the prolonged use of stimulant laxatives can induce dependency. The classical concept of dependency to a drug is characterised by specific features such as lack of control over intake, compulsive use and craving for the drug. The addiction to drugs usually occurs via the activation of dopaminergic systems after passage through the blood-brain barrier. Stimulant laxatives are not absorbed and do not pass the blood-brain barrier, so there is no pharmacologic basis for dependency. Moreover, the existence of "rebound constipation" after the laxatives are stopped has not been definitively established[6]. However, many constipated patients require a constant intake of laxatives to achieve normal (or what they believe to be "normal") bowel movements. Additionally, the abuse of laxatives has been reported in some patients; these cases of laxative misuse are often the result of psychological/psychiatric problems[53]. The use of SPS and bisacodyl is supported by Level I evidence, Grade B recommendation. It is not possible to provide graded recommendations for the other stimulant laxatives because placebo-controlled trials are lacking.

Softening laxatives

Softening laxatives make the stool softer by forming an emulsion of the faeces with lipids and water. Olive oil and sweet almond oil can function as softeners if their intake exceeds the absorptive capacity of the small intestine.

What evidence is there for the effectiveness of softening laxatives?

Docusate: Docusate is an anionic detergent that mixes aqueous and fatty components, thereby softening the stool; it may be administered orally or rectally through enemas or micro-enemas. When administered per rectum, docusate acts within 30 min.

Placebo-controlled trial of docusate: Two trials testing orally administered docusate yielded conflicting results. In the first trial, there was no difference in the stool frequency[54], but in the other study, a significant difference in stool frequency was observed ($P < 0.01$)[55].

Comparison of docusate with other laxatives: Psyllium was more effective than docusate for stool softening ($P < 0.04$)[56]. Therapy with docusate is supported by Level V evidence, Grade C recommendation.

Liquid paraffin (vaseline oil): Liquid paraffin is taken

orally and begins to take effect 6-12 h after administration. It acts by reducing the absorption of water and electrolytes. Its chronic use can damage the mucosal epithelium and lead to malabsorption of fat-soluble vitamins; aspiration can cause lipoid pneumonia. We did not find any randomised clinical trials in the literature on this product.

Glycerine: Glycerine, used as a suppository and mixed into enemas, is classified as a softening laxative, but its mechanism of action is unclear. It is assumed that, applied locally, glycerine produces tissue dehydration and irritation that in turn stimulate contractions of the rectum and defecation.

Serotoninergic enterokinetic agents

Serotonin (5-HT) is a critical component in the regulation of gut motility, visceral sensitivity, and intestinal secretion. Serotonin acts mainly on the 5-HT3 and 5-HT4 receptors expressed by enteric nervous system interneurones. Stimulation of the 5-HT4 receptor is responsible for excitatory effects such as the peristaltic reflex.

The 5-HT4 receptor agonists belong to several different classes of drugs. Cisapride is a substituted benzamide that acts as a partial 5-HT4 receptor agonist. Renzapride is a benzamide hydrochloride that is a full agonist of the 5-HT4 receptor, an antagonist of the 5-HT3 receptor, and a weak partial antagonist of the 5-HT2b receptor. Tegaserod is an aminoguanidineindole that is a 5-HT4/5-HT1 receptor partial agonist and a 5-HT2 receptor antagonist. It also has been shown to inhibit dopamine and noradrenaline transporters. Prucalopride is a dihydrobenzofurancarboxamide that is a selective 5-HT4 receptor agonist.

What evidence is there for the effectiveness of these drugs?

Placebo-controlled trials of serotoninergic enterokinetic agents: A recent systematic review of available controlled trials[57] showed that cisapride was more effective than a placebo in improving the gastrointestinal transit time. However, there was no evidence that cisapride use resulted in a global improvement of constipation-related symptoms compared to the placebo. It was concluded that cisapride use had no clear benefit and that the use of this drug could not be justified because of its cardiotoxic side effects. Indeed, in 2000, cisapride was withdrawn from the market because it had been associated with rare dose-dependent cardiac events, including lengthening of the QT interval, syncope, and ventricular arrhythmia in patients with predisposing conditions. These effects may be caused by the interaction of cisapride with the cardiac hER potassium channel. Although cisapride was withdrawn from the market, it can still be purchased online.

In a placebo-controlled trial, renzapride[58] was only marginally superior to a placebo in reducing the symptoms of constipation and did not improve the quality of life. This drug can cause ischemic colitis.

Large studies of tegaserod[59-61] and prucalopride[62-65] have been conducted that enrolled over 2500 and 2000 patients, respectively. Both drugs improved the stool frequency ($P < 0.001$), improved stool consistency, decreased the need for rescue medications, and reduced the symptoms of constipation. Tegaserod was previously approved in the United States but was not approved in Europe, except in Switzerland. It was withdrawn from the market in March 2007 because of an increased risk of cardiovascular adverse events (including myocardial infarction, unstable angina, and stroke) and is now only available for emergency use. The cardiovascular side effects may be related to vasoconstriction mediated by 5-HT1B receptors in the vascular wall[66].

Drugs with a higher selectivity for 5-HT4 receptors (e.g., prucalopride) may be able to minimise the incidence of cardiac side effects. In a small trial of patients with chronic noncancer pain suffering from opioid-induced constipation, prucalopride was found to be effective and safe[67]. The European Medicines Agency (EMA) approved the use of prucalopride in July 2009. The use of prucalopride is supported by Level I evidence, Grade A recommendation.

Prosecretory agents

Prosecretory agents stimulate the secretion of fluid into the intestinal lumen by activating intestinal chloride channels (lubiprostone) or the guanylate-cyclase receptors of enterocytes (linaclotide).

What evidence is there for the effectiveness of the prosecretory agents?

Placebo-controlled trials using lubiprostone: Two trials[68,69] showed that lubiprostone significantly increased the stool frequency, improved the stool consistency and reduced straining. Nausea was reported as a side effect. The Food and Drug Administration approved the use of lubiprostone for the treatment of adult patients with chronic idiopathic constipation in 2006. The EMA has not yet approved its use in Europe. The use of lubiprostone is supported by Level I evidence, Grade B recommendation.

Linaclotide: This drug has been reported to be effective in irritable bowel syndrome (IBS)[70]. In patients with chronic constipation[71,72], linaclotide significantly increased the stool frequency, improved the stool consistency and reduced straining. The health-related quality of life improved. Diarrhoea can be a side effect. The drug is currently being tested in a phase Ⅱ study.

Gastrointestinal μ-opioid receptor antagonists

Constipation is a side effect of opioid treatment that results from interference with the gastrointestinal μ-opioid receptors. Methylnaltrexone and alvimopan can increase the intestinal motility in patients taking opioid medications ($P < 0.001$) without neutralising the analgesic effect

of opioids[73-75].

One trial showed these drugs to be ineffective in patients with constipation-predominant IBS[76]. It therefore appears that gastrointestinal μ-opioid receptor antagonists are only effective against opioid-induced constipation. Level I evidence, Grade A recommendation regarding the ineffectiveness of this drug in patients with functional constipation.

Probiotics

Probiotics are orally administered living microorganisms that can reach and colonise the bowel. They are mainly prescribed to reduce the bloating and abdominal pain that accompany IBS.

How effective are probiotics in the treatment of chronic constipation?

Milk fermented with *Bifidobacterium lactis* reduced the symptoms of IBS but not constipation in 41 female IBS patients[77]. In women with "self-reported" constipation, probiotics reduced the severity of constipation (P = 0.003)[78]. *Bifidobacterium animalis* increased the stool frequency ($P < 0.01$) and improved the stool consistency in both healthy and constipated women[79]. A study involving 28 subjects reported that *Lactobacillus rhamnosus* + *Propiobacterium freudenreichii* improved the stool frequency but did not decrease the consumption of laxatives[80].

Placebo-controlled trials of probiotics in chronic idiopathic constipation are lacking. Probiotics can be considered for use in conjunction with other drugs in the treatment of chronic constipation. The use of probiotics to treat chronic constipation is supported by Level V evidence, Grade C recommendation.

Colchicine

Colchicine is effective in the treatment of a variety of inflammatory syndromes. In two controlled trials that enrolled a total of 72 patients, colchicine increased the stool frequency and reduced the consumption of laxatives[81,82]. The use of colchicine is supported by Level Ⅲ evidence, Grade C recommendation.

Procedures to empty the rectum and sigmoid colon

There is no controlled randomised trial in the literature addressing the chronic use of suppositories or enemas, which are commonly used for relief from occasional constipation and to empty the rectum in bedridden patients or those with impacted faeces.

Suppositories act by making the stool softer and by generating a foreign body stimulus that leads to the defecation reflex. The precise mechanism of action of transanal irrigation is unknown. Its efficacy may be related to a wash-out effect, and large volumes of liquid generate mass movements[83]. Irrigation volumes of less than 100 mL do not produce distension of the rectum. Without evacuation, the liquid and its solutes are absorbed from the rectum.

Hyperphosphatemia has been reported as a serious adverse event following the misuse of sodium phosphate enemas. A trial recently showed that transient mild hyperphosphatemia following the use of sodium phosphate enemas correlates with retention time but not with dose[84].

Transanal irrigation of the colon, with or without a rectal balloon catheter (Peristeen®), is a useful approach in patients with neurogenic bowel dysfunction secondary to spinal injury[85].

What is the evidence confirming the effectiveness of transanal irrigation in chronic constipation?

Based on the rate of colonic emptying, transanal irrigation appears to be more effective in patients with spinal cord damage than in those with chronic constipation[85].

Retrospective studies report the benefits of transanal irrigation for constipated patients. In one study of 16 patients, symptoms were reduced in 19% of patients suffering from constipation or obstructed defecation[86]. In another study of 37 patients, with a mean observation period of 4.5 years and a maximum observation period of 13 years, transanal irrigation was beneficial in 45% of patients with defecation disturbances[87]. Cazemier *et al*[88] consulted the database of enterostomal therapists for 12 patients for constipation and reported that after a mean observation period of 8.5 years, 42% of patients routinely used transanal irrigation, and 60% were satisfied with this method.

In patients with obstructed defecation, Gosselink *et al*[87] and Gardiner *et al*[89] reported, with colonic irrigation, an effectiveness of 65% and 57%, respectively. Recently, Christensen *et al*[90] presented the results of a long-term study on Peristeen® and reported positive effects in 31% of patients with slow transit constipation, in 43% of patients with obstructed defecation, and in no patients with indeterminate constipation. Retrograde irrigation for the treatment of chronic constipation is supported by Level V evidence, Grade C recommendation. Table 2 summarises the levels of evidence and the recommendation grade for various drugs.

At what point and on what grounds should we judge a treatment to be ineffective?

Generally accepted criteria to define the response to medical therapy have not yet been developed. To date, every clinical trial has defined its own endpoints in terms of the response to therapy.

A medical treatment should be considered unsatisfactory when a patient with good compliance does not report an appreciable improvement in the symptoms and quality of life after at least 4 wk of treatment at the full dose. To assess the therapeutic response, patients should be encouraged to keep a daily diary of their symptoms[91].

REHABILITATIVE TREATMENT

Dys-synergic defecation can be effectively treated by rehabilitative treatment (RT). Protocols vary among dif-

Table 2 Levels of evidence and grades of recommendation of medical treatment in chronic constipation

	Level of evidence	Grade of recommendation
Life style		
Physical exercise	V	C
Toilet training	V	C
Increased fluid intake	V	C
Bulking laxatives		
Insoluble fibre	III	C
Soluble fibre: Psyllium	II	B
Osmotic laxatives		
Lactulose	II	B
Sorbitol	V	C
Magnesium hydroxide/magnesium salts	V	C
Polyethylene glycol	I	A
Stimulant laxatives		
Sodium picosulfate, bisacodyl	I	B
Senna, aloe, cascara	V	C
Softening laxatives		
Docusate	V	C
Serotoninergic enterokinetics		
Tegaserod	I	A[1]
Prucalopride	I	A
Prosecretory agents		
Lubiprostone	I	B[2]
Linaclotide	I	B[3]
Gastrointestinal μ-opioid antagonists		
Methylnaltrexone	I (no effect)	A (not used)
Alvimopan	I (no effect)	A (not used)[2]
Probiotics	V	C
Colchicine	III	C
Procedures to empty the rectum-sigma		
Peristeen®	V	C

[1]Food and Drug Administration prescribing restrictions; [2]Not approved in Europe; [3]Phase II study. Adapted from: American College of Gastroenterology Chronic Constipation Task Force[184]; and Rao[249].

ferent centres, but all RT programs aim to improve defecation-related behaviour and restore a normal pattern of defecation with both instruments and educational devices.

Electrostimulation, kinesitherapy, biofeedback and volumetric rehabilitation can be used in various combinations to correct the dys-synergic behaviour of abdominal, rectal, and anal sphincter muscles and to improve rectal sensory perception.

RT requires a highly trained therapist and is time-consuming both for the therapist and the patient. Patients must therefore be strongly motivated.

When should RT be prescribed for obstructed defecation?

Functional obstructed defecation is the main indication for RT. Biofeedback is the treatment of choice for patients affected by pelvic floor dys-synergia. Three randomised controlled trials[92-94] have shown a success rate of approximately 70%[76] and a long-term success rate of approximately 50%[94]. Level I evidence, Grade A recommendation.

RT is also an effective therapy for organic diseases such as descending perineum syndrome, rectocele, recto-anal intussusception, rectal mucosal prolapse, rectal solitary ulcer syndrome not related to rectal prolapse, and second-degree sigmoidocele[95,96]. Level IV evidence, Grade C recommendation.

What is the recommended RT for obstructed defecation?

There are no universally accepted recommendations for RT, and there are no specific criteria to evaluate the efficacy of this intervention. The methods used such as biofeedback, kinesitherapy, electrostimulation and volumetric rehabilitation can differ greatly, resulting in a considerable variation in rehabilitation programmes among centres[97]. For this reason, the results of different studies may not be comparable[98-100].

Biofeedback is a technique based on the use of instruments to provide an information loop whose aim is to achieve operant conditioning[101]. Information on a physiological function (such as muscle contraction/relaxation) is translated into a visual or audio signal. When the execution of a muscle movement is correct, a signal is activated. Thus, erroneous functioning can be immediately corrected and the subject is conditioned to perform the correct contraction or relaxation movement. Some authors have studied the use of biofeedback combined with kinesitherapy for the pelvic and perineal muscles. The aim of the therapy in this setting is to teach the patient the correct sequence of contraction and relaxation of the striated muscles that is required for defecation[102-105]. It must be noted that these rehabilitative techniques have not been codified, they vary widely from one country to another, and they are supported by only one randomised trial[98]. Level II evidence, Grade B recommendation.

RT may be useful for improving rectal sensation when anorectal manovolumetry demonstrates rectal hyposensitivity in patients with obstructed defecation[106]. Such RT may be performed through biofeedback ("sensory retraining")[107] or volumetric rehabilitation using an inflated balloon or water enemas of decreasing volume and a probe to monitor muscle movement[108]. Neither biofeedback nor volumetric rehabilitation is supported by randomised controlled trials. Level IV evidence, Grade C recommendation.

Is RT the first therapeutic option?

Biofeedback, either alone or in combination with other rehabilitative techniques[102,103], is generally attempted only after pharmacological therapy has failed[95]. After drugs, however, rehabilitation is the treatment of choice in patients affected by obstructed defecation because there are no side effects. Even if RT fails, it will not have a deleterious effect on the patient's condition, and its results will not affect future decisions regarding therapy, including surgery[106,108,109].

Is RT more effective than drug therapy?

One randomised trial showed that biofeedback was su-

perior to laxatives in improving defecation in patients affected by pelvic floor dys-synergia[97]. It should be noted that the laxative dosage was not high in the control group, but the finding remains that rehabilitation reduced the need for laxatives. Level I evidence, Grade B recommendation.

What factors may influence the efficacy of RT?

There is no general agreement as to what factors may predict or influence the outcome of RT[93,105,110]. In one study, no correlation was found between specific conditions (rectocele, ano-rectal intussusception, descending perineum and sigmoidocele) and the efficacy of biofeedback in patients with dys-synergic defecation[111]. Level Ⅳ evidence, Grade B recommendation. In a more recent study, harder stools, shorter duration of laxative use, higher rectal pressure while straining and prolonged balloon expulsion independently predicted a positive outcome for RT[112]. Level Ⅲ evidence, Grade C recommendation.

Nevertheless, significant anatomic damage, severe psychiatric or neurological disease, poor patient compliance, and poor patient–physiotherapist interactions could pose major obstacles to the success of RT[97,113]. Level Ⅲ evidence, Grade C recommendation.

Does surgery make RT superfluous?

No, the experience suggests that RT, even if it does not achieve satisfactory function, can improve pelvic floor muscle tone and coordination and can contribute significantly to a positive outcome in ano-rectal surgery[114]. Level V evidence, Grade C recommendation.

How should patients who do not respond to RT be managed?

It is not clear how non-responsive patients should be managed because a validated, universally accepted set of criteria regarding the response to RT has not yet been developed. If the clinical grounds for prescribing RT were appropriate (presence of dys-synergic defecation and/or inadequate propulsive forces) and no negative predictive factors are present, the failure of a patient to respond to RT should raise the suspicion of significant organic damage (e.g., a rectocele or rectal intussusception) and lead to the evaluation of surgical options[115]. In fact, in patients with a rectocele and/or ano-rectal intussusception, one of the prerequisites for surgery to correct obstructed defecation is the failure to respond to RT[115]. Level Ⅱ evidence, Grade C recommendation.

In one report on a small number of patients, sacral neuromodulation was found to be effective[116]. Level Ⅳ evidence, Grade C recommendation.

Should RT be prescribed before or after ano-rectal surgery?

Although it is difficult to discriminate between patients who will derive some benefit from RT and those who instead will require surgery, the generally accepted procedure is to begin with RT[117] and, if this proves ineffective, to then consider surgery[115]. Level Ⅱ evidence, Grade B recommendation.

There are no clear guidelines to help the clinician decide between the approaches of "rehabilitation-surgery" and "rehabilitation-surgery-rehabilitation". RT should certainly be prescribed if the outcome of ano-rectal surgery is unsatisfactory[117]. In cases involving large rectoceles, anal fissures or severe anatomical anorectal changes, where it is unlikely that RT will have any direct effect, rehabilitative therapy should be prescribed after surgery[118]. Level V evidence, Grade C recommendation.

Concerning the efficacy of RT following surgery, only a handful of reports on patients who underwent RT for obstructed defecation after ano-rectal surgery have been published, each involving a different protocol. RT has been successful in treating persistent symptoms of obstructed defecation after stapled trans-anal rectal resection (STARR)[119,120], hemorrhoidectomy, and surgery for mucosal rectal prolapse[121]. Level V evidence, Grade C recommendation.

What are the medium- and long-term effects of RT?

Lasting improvement has been observed in patients with dys-synergic defecation (confirmed clinically and by manometry) up to 2 years after RT[92,122-124]. Level I evidence, Grade B recommendation.

SURGICAL TREATMENT FOR SLOW-TRANSIT CONSTIPATION

Colectomy

What are the conditions and selection criteria for colectomy?: There are no randomised trials focusing on the selection of constipated patients for surgery. The most commonly described selection criteria for segmental, subtotal or total colectomy are (Level V evidence, Grade C recommendation): (1) ≤ 2 weekly defecations[125-131]; (2) duration of symptoms (mean 5-17 years)[130,132]; (3) the presence of symptoms such as abdominal bloating or pain, nausea, and vomiting that have a significant impact on the patient's quality of life[110,113,130,132,117]; (4) failure of behavioural, dietetic, pharmacological and RTs to improve the symptoms[109-114,133]; (5) radiological evidence of slow-transit constipation[130,131,133-138]; (6) exclusion of organic or functional pelvic floor disorders (obstructed defecation, Hirschsprung's disease) based on defecography and ano-rectal manometry[131,136-138]; (7) exclusion of upper gastrointestinal tract dysmotility based on functional (manometric, scintigraphic) examinations, if dyspeptic symptoms are present[137,139-141]; and (8) normal results of psychological evaluation (patients with psychological disorders tend to show poor results after surgery for constipation)[136,142-145].

Total or subtotal colectomy with ileorectal anastomosis

What are the levels of evidence and grades of recommendation for this procedure?: No randomised or controlled studies have been published on this proce-

dure. Level V evidence, Grade C recommendation.

What is the success or satisfaction rate following surgery?: Definitive conclusions regarding the effectiveness of surgery cannot be drawn. The methods used to assess the outcome of surgery and patient satisfaction vary greatly. Among 39 published studies, 6 did not define the criteria used, in 17 the assessment was based solely on patient feedback, and in 8 the conclusions were based on the results of post-operative functional tests. Only in 8 studies was the success of surgery determined from functional results and the patient's evaluation. The data collection methods also differ between the studies; they were not defined in 15 of the 39 studies reviewed, whereas a questionnaire was used in 15 studies; only in 9 studies was a post-operative interview conducted. The overall rate of success or satisfaction documented in 39 studies involving 1423 patients was 86% (39%-100%)[125,129,130,133,136,146-149].

What are the post-operative morbidity and mortality rates?: The overall post-operative morbidity in 25 series was 20% (2%-71%)[125,129,130,134,149], and the mortality documented in 26 studies was 2.6% (0%-15%)[125,130,134,146].

Post-operative complications

What is the incidence of small bowel obstruction, diarrhoea, faecal incontinence and abdominal pain, and what is the re-operation rate?: The complication rates were (mean and range): (1) small bowel obstruction, evaluated in 26 series with 913 patients: 18% (2%-71%)[125,129,130,134,146]; (2) chronic diarrhoea, in 19 series with 843 patients: 14% (0%-46%)[130,136,146,148]; (3) faecal incontinence, in 21 series with 913 patients: 15% (0%-52%)[125,129,130,136,146,148]; (4) abdominal pain, in 19 series with 839 patients: 35% (0%-90%)[125,129,130,136,143,146,148]; and (5) re-operation rate, evaluated in 5 studies with 965 patients: 14% (0%-50%)[125,130,134,149].

What are the functional results after surgery, in terms of daily bowel movement rates and recurrent constipation?: In 26 studies[125,129,130,133,146,148] involving 1047 patients and with a mean follow-up period of 44.8 (12-180) mo, the rate of bowel movements reported by the patients was 2.8 times per day, whereas recurrent constipation was reported by 9% (0%-33%) of 683 patients in 17 series[129,130,136].

How often is a permanent stoma the only solution for a patient?: The incidence of permanent stoma placement as a therapy of last resort was 5% (0%-28%) in 27 studies involving 930 patients[125,130,150].

What prognostic factors have been identified?: Preservation of the ileocaecal valve and caecum during isoperistaltic anastomosis resulted in a higher rate of persistent or recurrent constipation[145,146,150,151]. One controlled study[146] reported better results in 45 patients who underwent ileosigmoid anastomosis compared with 34 patients who underwent isoperistaltic caecorectal anastomosis (93% *vs* 73%). Level Ⅲ evidence, Grade C recommendation. (1) Negative prognostic factors described in the literature include the onset of constipation in adulthood or after pelvic or intestinal surgery[151-153]; (2) Positive prognostic factors include childhood onset and "lifelong" duration of symptoms[154,155].

Is autonomic neuropathy an indication for surgery?: A higher rate of post-operative complications (especially small bowel obstruction) and the persistence of pre-operative symptoms (abdominal pain and bloating) have been reported in patients with autonomic neuropathy[141]. Therefore, some authors consider autonomic neuropathy to be a contraindication to surgery[138]. Level V evidence, Grade C recommendation.

Can laparoscopy yield better results than surgery?: Four studies without controls and one evaluating a "hand-assisted" technique have been published, but these were primarily feasibility studies[129,134,136,156,157].

Segmental colectomy

Can segmental colectomy lead to better functioning?: It appears that if the decision to undertake segmental colectomy is based on radiologically demonstrated segmental colonic slow transit, good results can be achieved in 82%-96% of patients[136,158,159]. Without this evaluation, the failure rate is 100%[151,160]. Level V evidence, Grade C recommendation.

Subtotal colectomy with antiperistaltic caecoproctostomy (Sarli's procedure)

Can this procedure lead to better results than conventional total or subtotal colectomy?: In the literature, 3 series[126,157,161] have reported on a total of 43 patients in whom a subtotal colectomy with caecal preservation and antiperistaltic cecorectal anastomosis was performed. These were non-randomised but controlled studies, with subjects undergoing ileorectal anastomosis. Level Ⅲ evidence, Grade C recommendation. Patients reported less faecal incontinence (documented by the Wexner score), less use of anti-diarrhoeal drugs, fewer defecations per day, more consistent faeces and a better quality of life after surgery. After 4.5 years (range 2-7) of follow-up, the mean daily number of bowel movements was 2.5, and the success rate of the surgical procedure was 88% (65% in patients with ileorectal anastomosis).

Does this procedure have a lower incidence of complications?: The cumulative post-operative complication rate was 9% (in 22 patients); 2 (1%) patients experienced small bowel obstruction, but none complained of diarrhoea[126,157,161].

Malone antegrade continence enema

What patients are candidates for this procedure?: Although good results have been reported recently in

adults, Malone antegrade continence enema (MACE) is most successful in paediatric patients[162,163] or in patients with neurological diseases such as myelomeningocele, cerebral or spinal cord injuries, or Hirschsprung's disease[163-169]. The greatest improvement in the quality of life was observed in patients with concomitant faecal incontinence[168]. Level V evidence, Grade C recommendation.

What is the level of evidence and recommendation grade for this procedure?: The level of evidence and recommendation grade cannot be determined because there are no randomised or controlled studies on MACE in the literature[162-172].

What is the success or satisfaction rate with the Malone procedure?: The methods of assessing the overall success or satisfaction rate vary widely among studies. Among 7 studies, the criteria were not clearly defined in one, success/satisfaction was based on the patient's judgement in another, and in 5 the outcome was evaluated based on functional results and the patient's judgement. The data collection methods also varied; they were based on a questionnaire and interview in 4 studies, whereas an interview was conducted in only 3 studies. With a mean follow-up period of 44 (range 12-78) mo, the overall success or satisfaction rate, documented in 7 studies on 67 patients, was 74% (50%-100%)[164-166,169-172], but within 3 years, the MACE procedure was replaced by other therapies in 50%-75% of cases[165,171].

What complications have been observed?: The main complication of this procedure is stenosis of the stoma (8%-50%)[163,165,166,169-171].

Sacral nerve stimulation

What is the level of evidence and the recommendation grade for this procedure in patients with constipation?: One double-blinded, placebo-controlled crossover study in 2 patients[173] and 7 non-randomised studies[116,174-179] have been published, but 3 of these studies[173-175] probably used the same patients. One of these trials was presented only in the form of an abstract at Digestive Diseases Week in 2007[177] and for this reason was not taken into consideration in the calculation of the mean success rate. In this report by Kamm *et al*[177], the success rate of a temporary implant was 66% (n = 67), and all patients with a permanent implant continued to show good results after 12 mo of follow-up.

In a recent prospective study at five European sites, sacral nerve stimulation (SNS) was effective among patients with idiopathic slow and normal transit constipation resistant to conservative treatment. The primary end points were increased defecation frequency, decreased straining and decreased sensation of incomplete evacuation ($P < 0.001$)[179] (Level Ⅲ evidence, Grade C recommendation).

What is the mean success rate of percutaneous nerve evaluation followed, if indicated, by the insertion of a permanent pacemaker?: The overall success rate of percutaneous nerve evaluation, as documented in 4 studies involving 86 patients, was 60% (25%-75%)[174,176,177,179]. In 2 studies[116,175] involving a total of 12 patients who received permanent pacemakers, the reported success rates were 100% and 75% after a median follow-up of 11 and 8 mo, respectively. Finally, the multicentre prospective study coordinated by Kamm *et al*[179] reported that after a median of 28 mo (range 1-55), the frequency of defecations increased from a baseline of 2.3 evacuations per week to 6.6 evacuations per week.

SURGERY FOR OBSTRUCTED DEFECATION

The management of patients affected by obstructed defecation can be challenging because of the frequent association of anatomical and functional anomalies, which makes it difficult to distinguish between the causes and consequences of excessive strain[180,181]. The complexity of the syndrome and the range of available treatments make the outcome of the therapy unpredictable[182]. Surgery is usually considered for patients with reparable anatomical defects, concomitant pathologies, or symptoms that severely impact their quality of life[183].

Indications for surgery in constipation arising from obstructed defecation

Surgical treatment is indicated in cases of reparable anatomical defects, severe symptoms, symptoms leading to a poorer quality of life, or concomitant pathologies[183,184]. Level V evidence, Grade C recommendation.

What criteria are there for evaluating treatment efficacy (severity score, defecography, quality of life)?

The obstructed defecation syndrome (ODS) score is a tool designed to evaluate patients suffering from *pure* outlet obstruction without slow transit or mixed forms of constipation. The ODS provides an index of the disease severity and can be used to monitor the efficacy of therapy[182]. Level Ⅲ evidence, Grade C recommendation. Other tools, such as the Constipation Scoring System[185] and the KESS Score[186], are not specific for ODS but can be employed to study other forms of constipation; some items in these scores are not influenced by the therapy[185].

There are two approaches - abdominal (rectopexy) and trans-anal (STARR or Delorme transrectal excision) - to surgically correct internal intussusception: Which is recommended on the basis of the clinical evidence?

The results of rectopexy are uncertain. Some studies report the persistence or worsening of constipation and difficulty in emptying the rectum in approximately 50% of cases, although the prolapse is corrected in almost all patients[187]. Other studies report that resection and rec-

topexy improve the symptoms relating to intussusception and coexisting anatomical and functional pathologies of the pelvic floor, such as enterocele, solitary ulcer of the rectum, incontinence and descending perineum syndrome[188]. Level V evidence, Grade C recommendation.

Colonic resection and rectopexy can reduce intussusception in 100% of cases, restore anal muscle tone (P = 0.002), reduce the descending perineum ($P < 0.001$) and accelerate colonic transit ($P < 0.001$) with stable results over time (based on a 5-year follow-up)[188]. Level V evidence, Grade C recommendation.

Sutureless rectal mobilisation, suture rectopexy, and mesh rectopexy: Which is better?

In a multicenter randomised study of 252 patients, an actuarial analysis demonstrated a significant difference in 5-year recurrence rates between no-rectopexy and rectopexy groups (8.6% *vs* 1.5%) (log-rank, $P = 0.003$)[189]. Level V evidence, Grade C recommendation.

A ventral instead of posterior mobilisation and fixation of the mesh have recently been advocated and popularised. Excellent results have been claimed, but no randomised comparative trials have been conducted thus far[190-193]. Level V evidence, Grade C recommendation.

Laparoscopy or laparotomy for rectopexy?

Although there are no studies comparing the two different approaches, there is a tendency in the literature to perform this operation laparoscopically because of the potential of this approach to shorten hospital stays, decrease the incidence of abdominal wound complications and improve cosmesis.

A trial assessed the quality of life in ODS patients comparing STARR with biofeedback and reported that the STARR was better ($P < 0.0001$)[115]. Level II evidence, Grade C recommendation.

Which approaches to repair a rectocele (trans-anal, trans-vaginal, perineal, etc.) can be recommended on the basis of evidence based medicine?

The indications for surgery and the choice of procedure are still being debated, and a clear correlation between the correction of the anatomical problem and the improvement of symptoms has not yet been demonstrated[194]. Surgery should only be considered when conservative therapy has failed. Options include trans-vaginal posterior colporraphy and trans-rectal or trans-perineal repair[183]. In terms of an improvement in symptoms, Arnold *et al*[195] did not find any difference between the trans-anal and the trans-vaginal approaches. Level V evidence, Grade C recommendation. Even when the surgical repair has been correctly performed, 30%-72% of patients still experience difficulties in defecating[196]. Level V evidence, Grade C recommendation. Post-surgical complications include faecal incontinence and sexual dysfunction[197,198].

Is there a technique that can be considered the gold standard for the treatment of ODS?

Numerous surgical procedures using different approaches (abdominal, vaginal, trans-anal or perineal) are available for the treatment of ODS, but none has been identified as the gold standard[115]. Level II evidence, Grade B recommendation.

Can obstructed defecation resulting from rectocele/intussusception also be associated with slow-transit constipation?

Slow colonic transit is often observed in patients with a symptomatic rectocele[198,199]. The obstructed defecation in these patients does not appear to exclusively result from the rectocele (Level V evidence). Although improved rectal emptying may be observed after surgical correction, this effect is not likely to affect colonic function. It has been demonstrated that patients with poor functioning after repair of the rectocele still have a prolonged colonic transit time[200]. Furthermore, patients with a slow transit time before the operation show little improvement after surgery[198] (Level V evidence).

Can STARR be effective in the treatment of patients with ODS who fail to respond to medical and RT?

The efficacy and safety of STARR has been demonstrated in ODS patients in whom 5-10 sessions of biofeedback therapy (BF) failed[115]. Level II evidence, Grade B recommendation. This study showed a reduction of the ODS score in 81.5% of patients after STARR (one-year follow-up) compared with a reduction of 13% after BF (difference between the two groups: 48.1%, $P < 0.0001$, 95% CI: 30.1%-66.2%). This study had several drawbacks, however, including the small number of participants enrolled, a 50% dropout rate in the BF group, and the fact that a surgical approach was compared with a non-surgical one.

Can STARR improve the quality of life in patients with rectal intussusception or a rectocele?

The STARR procedure can significantly improve the quality of life in patients suffering from ODS arising from rectal intussusception or a rectocele compared with biofeedback ($P < 0.0001$)[115]. However, other options, such as pelvic floor rehabilitation or the internal Delorme procedure[201], could be considered instead to prevent any potential risk associated with the stapling procedure. Level II evidence, Grade B recommendation.

In cases of ODS arising from rectal intussusception or rectocele, can stapled trans-anal prolapsectomy with perineal levatorplasty alleviate the symptoms?

Boccasanta *et al*[202] compared the stapled trans-anal prolapsectomy with perineal levatorplasty (STAPL) and STARR procedures in a randomised trial. All of the symptoms relating to obstructed defecation improved following both procedures. After a 20-mo follow-up, the

results were still good in 76% of the STAPL patients and in 88% of the STARR patients. The authors nonetheless concluded that STARR is preferable because of the lower post-operative pain ($P < 0.001$), reduced rectal sensitivity ($P < 0.017$), absence of dyspareunia ($P < 0.018$), and a more marked reduction in the rectocele. Level I evidence, Grade B recommendation.

What procedure should be performed in cases of complete rectal prolapse?

Abdominal rectopexy with sigmoidectomy and plain rectopexy with mesh are safe and effective procedures for the treatment of complete rectal prolapse[202]. Level Ⅱ evidence, Grade B recommendation. Only one prospective randomised trial has compared abdominal rectopexy and sigmoidectomy (group Ⅰ) with rectopexy and a polyglycolic acid mesh (group Ⅱ). After correction of the prolapse, 8/11 patients in group I suffering from incontinence and 10/12 in group Ⅱ who had incontinence improved. Six months after surgery, the constipation had disappeared in 3 and 7 patients from groups I and Ⅱ, respectively, but 5 other patients from group Ⅱ required a colectomy within one year after the operation because of severe constipation[203].

Is abdominal rectopexy with sigmoidectomy associated with higher rates of morbidity than simple rectopexy in patients with complete rectal prolapse? Should this procedure be performed in cases of slow transit constipation or only in patients with dolicocolon?

Sigmoid resection with rectopexy is effective in reducing post-operative constipation arising from outlet obstruction without increasing the rate of morbidity[201]. Level Ⅱ evidence, Grade B recommendation. Rectopexy with sigmoid resection can improve the colonic transit ($P < 0.001$)[196], even in the presence of dolicocolon (Level V evidence, Grade C recommendation).

Is SNS effective for the treatment of patients with ODS constipation?

SNS may be effective in the treatment of chronic constipation when other approaches have failed[204]. In a recent prospective study at five European sites, SNS was effective in patients with idiopathic slow and normal transit constipation who failed conservative treatment. In this study, the primary end points were increased defecation frequency, decreased straining and decreased sensation of incomplete evacuation ($P < 0.001$)[179] (Level Ⅲ evidence, Grade C recommendation).

When surgery is indicated for solitary rectal ulcer syndrome, what procedure should be adopted?

Solitary rectal ulcer may be associated with paradoxical contraction of the puborectal muscle, recto-anal intussusception, rectal prolapse and descending perineum syndrome. Treatment of this condition must be conservative. Surgery can be considered for patients with full-thickness rectal prolapse or intractable haemorrhage, but the degree of continence and constipation and the risks of surgery must be carefully assessed in each individual patient, and the patient's preferences regarding treatment should be taken into account. The procedure selected must be safe and balance the risk of morbidity with an acceptable recurrence rate[183,205,206]. Level V evidence, Grade C recommendation. The surgical options include repair of the rectal prolapse with or without resection of the lesion, although the long-term results of this procedure were found to be uncertain. Anterior resection and proctocolectomy have shown satisfactory long-term results[207].

When is surgery indicated for sigmoidocele?

Surgery is indicated for symptomatic patients with third-degree sigmoidocele (below the ischiococcygeal line) or patients who require other pelvic surgery with an abdominal or vaginal approach (hysterectomy, rectal prolapse, rectocele repair). The surgery consists of sigmoid resection and rectopexy with obliteration of the Douglas pouch. Jorge *et al*[208] documented an improvement of the symptoms in 100% of patients undergoing this procedure, compared with 33% of patients who were treated conservatively, after a mean follow-up of 33 mo. Level V evidence, Grade C recommendation.

What is the best surgical treatment for megarectum with or without megacolon?

There has been considerable debate regarding the surgical treatment for megarectum. In megarectum with megacolon, colectomy and ileo-rectal anastomosis is the procedure with the best functional results and the lowest morbidity[209]. In patients who do not experience satisfactory results, total proctocolectomy and ileo-pouch-anal anastomosis is the treatment of choice to avoid permanent ileostomy[210,211] (Level V evidence). In idiopathic megarectum, proctectomy and colo-anal anastomosis with or without colonic reservoir has shown good results; defecation and faecal continence were satisfactory in 72% of patients[212,213] (Level V evidence). The Duhamel procedure is less successful for the treatment of idiopathic megarectum than for Hirschsprung's disease[214]. In idiopathic megarectum, this procedure is associated with the persistence of symptoms and often with the need for a repeat operation[215]. It has been reported that rectoplasty with vertical reduction of the rectum and sigmoid resection results in a significant improvement in the frequency of defecation, a reduced consumption of laxatives or enemas, and satisfaction with surgery in 83% of the patients[216] (Level V evidence, Grade C recommendation).

SURGERY FOR OBSTRUCTED DEFECATION WITH ASSOCIATED PELVIC DISEASES

How often is obstructed defecation associated with pelvic organ prolapse?

A posterior colpocele in pelvic organ prolapse (POP)

may be linked to anatomical conditions such as a rectocele, enterocele or sigmoidocele[217]. Of POP patients, 24%-52% complain of difficulties in defecation[218]. Straining is more common in women with prolapse (61% *vs* 4%; $P < 0.001$)[219].

Is there a correlation between POP and chronic constipation?

The literature is sparse on this point. In a study of 302 patients with urinary incontinence and/or POP, Jelovsek *et al*[220] concluded that chronic constipation plays no significant role in the etiology of the prolapse (Level Ⅳ evidence). Soligo *et al*[221] found that the prevalence of constipation was 33% (95% with obstructed defecation) in 786 women suffering from uro-gynecologic dysfunctions. A significant correlation was noted between constipation and posterior genital prolapse (Level Ⅳ evidence). Similar data have been reported by other authors[222-224].

How does the physiopathologic correlation between POP and obstructed defecation contribute to posterior colpocele and rectocele?

At present we do not know whether constipation is a symptom caused by anatomic functional defects of the pelvic floor[221], or whether it is the cause of static and dynamic changes in the pelvis. Pudendal neuropathy arising from stretching of the pudendal nerve while straining in patients with chronic constipation[225] (Level Ⅲ evidence) may explain the prolapse of the posterior wall and the overall weakening of the pelvic floor[226,227] (Level Ⅳ evidence). According to DeLancey[226], the tonic contraction of the levator ani, the perineal membrane and the endopelvic fascia provide the main support for the posterior vaginal wall. Under physiologic conditions, the levator ani has a double vector. The muscle exerts forward force (closing the vaginal walls) and then extends downward to the perineal body, supported by the perineal membrane, which anteriorly is anchored to the ischiopubic branches. This compensating balance eliminates any traction on the endopelvic fascia (corresponding to the middle third of the vagina or DeLancey level Ⅱ). Pudendal neuropathy reduces the strength of the levator ani, and the downward vector will tend to be toward the posterior vaginal wall rather than the perineal body. A weak endopelvic fascia leads to posterior vaginal wall prolapsed, which may explain both the high and low rectoceles involving the middle vagina and the perineal body, respectively.

What are the most recent anatomical and functional developments in pelvic reconstructive surgery?

Increasingly, prostheses are replacing fascial surgery in the treatment of POP. The synthetic prosthesis is set in a tension-free position and connected to structures such as the obturator membrane, the arcus tendineous of the pelvic fascia, the sacrospinous ligaments or the perineal body. The use of a synthetic mesh has resulted in a marked decrease in recurrence (from 29% in fascial reconstructions to 9% with synthetic mesh), and a significant increase in erosion from 0.7% with the re-absorbable mesh to 10.2% with the synthetic mesh[228] has been observed. Level Ⅱ evidence, Grade B recommendation. However, the efficacy and safety of the prostheses used for the posterior vaginal wall have not yet been established[228] (Level Ⅱ evidence).

Surgical treatment for rectocele has been evaluated in 4 randomised studies (Cochrane Review, 2007[229-233]. The transvaginal approach was associated with the lowest number of recurrences, the use of biologic prostheses did not reduce the recurrence rate, and there was no significant difference between trans-anal and transvaginal procedures in terms of effects on defecation. Level I evidence, Grade A recommendation.

Does obstructed defecation improve after the correction of a posterior colpocele with mesh?

Few studies have considered the effect of surgery for posterior colpocele on posterior compartment dysfunction. The improvement reported in 24%-28%[234,235] of cases in two studies was not based on an adequate scoring system (Level V evidence). The presumption that a dysfunction such as constipation can be treated simply by correcting an anatomic defect is probably incorrect. Approximately 30% of patients with obstructed defecation also complain of slow-transit constipation[236] (Level V evidence). We do not yet understand the pathophysiology of these conditions, particularly with respect to the role of the CNS and ENS. The severity of the obstructed defecation is also not correlated with the results of the pelvic organ prolapse quantification[237-239] or to the anatomic-functional data provided by defecography[222] (Level V evidence, Grade C recommendation).

What is the recommended surgical procedure for post-hysterectomy voltocele?

Voltocele affects 18.2% of women with genital prolapse after hysterectomy. In 72% of these patients, there is also an anterior (cystocele) or posterior colpocele (rectocele or enterocele)[240,241]. The incidence of enteroceles after hysterectomy is between 0.1% and 16%[242]. Voltocele is caused by damage to the supporting uterosacral and cardinal ligaments. Enterocele, a herniation of the Douglas pouch between the vagina and the rectum, is caused by damage to the perineal membrane, which supports the posterior vaginal wall and connects the ischiopubic branches to the perineal body (Level V evidence)[243,244]. The levator ani muscle is also important because it is connected to the middle of the vagina by the endopelvic fascia. Three procedures designed to prevent enterocele and posterior colpocele after hysterectomy, i.e., the Moschcowitz method, the McCall method, and simple closure of the Douglas pouch, were compared in a prospective randomised study with 3 years of follow-up, and the best results were obtained with the McCall operation[245] (Level Ⅱ evidence, Grade B recommendation).

Many techniques have been proposed to correct a

voltocele associated with an enterocele. The abdominal approach with mesh sacrocolpopexy and Douglas obliteration repairs the anatomic defect in 90% of cases[246-248]. In a Cochrane review of 22 controlled randomised studies, abdominal sacrocolpopexy appeared to be superior to the transvaginal approach (vault sacrospinous fixation), with fewer recurrences and less dyspareunia[229], but it is a longer and more painful procedure that involves a longer hospital stay and higher costs[241]. Level I evidence, Grade A recommendation. No data are available on the effects of these procedures on constipation.

APPENDIX

This paper is the second part of the consensus statement of the AIGO/SICCR regarding the diagnosis and treatment of chronic constipation and obstructed defecation. This section will focus on the treatment of this condition.

The first part of the paper was published in the *World Journal of Gastroenterology* 2012 April 14 (ISSN 1007-9327) and describes the materials and methods used to generate these recommendations. Similar to that paper, this article presents the results in a "question-and-answer" format.

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REVIEW

What we should know about portal vein thrombosis in cirrhotic patients: A changing perspective

Francesca Romana Ponziani, Maria Assunta Zocco, Matteo Garcovich, Francesca D'Aversa, Davide Roccarina, Antonio Gasbarrini

Francesca Romana Ponziani, Maria Assunta Zocco, Matteo Garcovich, Francesca D'Aversa, Davide Roccarina, Antonio Gasbarrini, Internal Medicine and Gastroenterology, Policlinico A. Gemelli, Catholic University of Rome, largo A. Gemelli, 800168 Rome, Italy

Author contributions: Ponziani FR wrote and revised the article; Zocco MA wrote and revised the article; Garcovich M revised the article; D'Aversa F contributed to literature collection; Roccarina D revised the article; Gasbarrini A revised the article.

Correspondence to: Dr. Francesca Romana Ponziani, MD, Internal Medicine and Gastroenterology, Policlinico A. Gemelli, Catholic University of Rome, largo A. Gemelli, 800168 Rome, Italy. francesca.ponziani@yahoo.it
Telephone: +39-6-30156018 Fax: +39-6-30155923
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Abstract

Portal vein thrombosis (PVT) is one of the most common complications occurring during the natural course of liver cirrhosis. Even though PVT is often asymptomatic, the worsening of liver function, an unexpected episode of gastrointestinal bleeding or ascitic decompensation may be landmarks of PVT development. Beyond these clinical manifestations, it is debated whether PVT really has an impact on liver cirrhosis natural history or rather represents only one of its consequences. Probably PVT development should not only be considered as a matter of impaired blood flow or pro-coagulation tendency. On one hand, PVT seems a consequence of the worsening in portal vein outflow due to the increased hepatic resistance in cirrhotic livers. On the other hand, vascular microthrombosis secondary to necroinflammation may cause liver ischemia and infarction, with loss of hepatic tissue (parenchymal extinction) which is replaced by fibrotic tissue. Therefore, PVT might also be considered as the overt manifestation of the liver fibrosing process evolution and anticoagulant therapy may thus have microscopic indirect effects also on the progression of liver disease. At present, a connection between PVT development and the progression of liver fibrosis/cirrhosis has not yet been demonstrated. Nevertheless, it is not clear if PVT development may worsen cirrhotic patients' outcome by itself. Some authors tried to assess liver transplant benefit in PVT cirrhotic patients but data are contrasting. In this review, we will try to answer these questions, providing a critical analysis of data reported in literature.

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Key words: Portal vein thrombosis; Liver cirrhosis; Liver fibrosis; Microthrombi; Survival

Peer reviewers: Erwin Biecker, MD, PhD, Department of Gastroenterology and Hepatology, Helios Klinikum Siegburg, 53343 Siegburg, Germany; Marco Senzolo, MD, PhD, Gastroenterology Unit, Department of Surgical and Gastroenterological Sciences, University-Hospital of Padua, Via Giustiniani 2, 35136 Padua, Italy

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INTRODUCTION

Portal vein thrombosis (PVT) is a well-known complication occurring during the course of liver cirrhosis, frequently in the advanced stages. It has been demonstrated that the alteration of blood flow within the portal vein plays an important role in PVT development, together with inherited or acquired pro-thrombotic disorders.

Over the past few years, PVT diagnosis has been constantly increasing due to the amelioration of diagnostic imaging, with partial PVT being now more easily recognizable (Figure 1).

When PVT occurs in a cirrhotic patient, it is always considered a harmful event, because the increase of portal pressure and impaired liver vascularization may lead to gastrointestinal bleeding, worsening of liver function, and ascitic decompensation.

PVT is a matter of concern, especially regarding its treatment. Since PVT occurrence often represents a precipitating event during the course of cirrhotic disease, at the moment of the diagnosis every clinician will try to resolve the obstruction. However, it is unclear whether it could be beneficial or not to treat asymptomatic patients, and which treatment could have the best risk/benefit ratio in cirrhotics. Although anticoagulants seem to be the most used treatment in the last few years, there is at present no consensus regarding the dose and duration of treatment[1].

Some cardinal questions regarding PVT still remain unanswered: as PVT occurs often in the advanced stages of liver disease, what is the connection between PVT development and the progression of liver fibrosis/cirrhosis? Does PVT really have an impact on liver cirrhosis natural history or is PVT only one of its consequences?

In this review we will try to answer to these questions, on the basis of the evidence reported in literature. We intend to point out the question if there really is a connection between PVT development, which often occurs in the advanced stages of liver disease, and histological progression of fibrosis.

PREVALENCE AND CAUSES

Among all cases of PVT, cirrhosis is the underlying cause in 22%-28%[2,3]. Studies based on ultrasonography have reported a prevalence of 10%-25%, excluding neoplastic thrombosis[4-7]. The prevalence of PVT increases with the severity of cirrhosis, being approximately 1% in patients with compensated cirrhosis[8] and rising to 8%-25% in candidates for liver transplantation (LT)[5]; evidence for hepatic vein thrombosis was found in 70% of livers explanted at a stage of advanced cirrhosis[9]. This large variability is due to the fact that PVT is often asymptomatic, therefore its frequency may be underestimated. Indeed, in the majority of patients with cirrhosis, PVT is diagnosed as an incidental finding, often during routine ultrasound evaluation. However, when an episode of decompensation such as the development of ascites or gastrointestinal bleeding occurs, the presence of PVT should always be suspected and investigated[10,11]. At the time of evaluation for LT[12], at LT[13] or at surgical porto-systemic shunting[14], the model for end-stage liver disease (MELD) and Child-Pugh scores seems to be higher in patients with PVT than in those without.

At the origin of PVT development, there is always one or more of the following reasons, which are included in the "Virchow triad": an impaired portal blood outflow (liver architectural changes of liver cirrhosis increase the resistances to flow), a tendency to hypercoagulability, or vascular damage, together with the development of hepatocellular carcinoma[15-17]. In cirrhotic patients, some of these factors may have a greater impact on PVT development (Table 1). Zocco *et al*[18] showed that portal flow velocity is the most important predictive factor for PVT development, especially when portal flow velocity is lower than 15 cm/s at Doppler ultrasound evaluation. It has been reported that when cirrhotic patients develop a hepatocellular carcinoma, PVT incidence rises to 10%-40%[19]. However, although the evidence of a pro-thrombotic condition is not frequent in cirrhotic patients, it should not be a priori excluded.

In a recent study including 136 consecutive cirrhotic outpatients (24.2% diagnosed with PVT), a significant

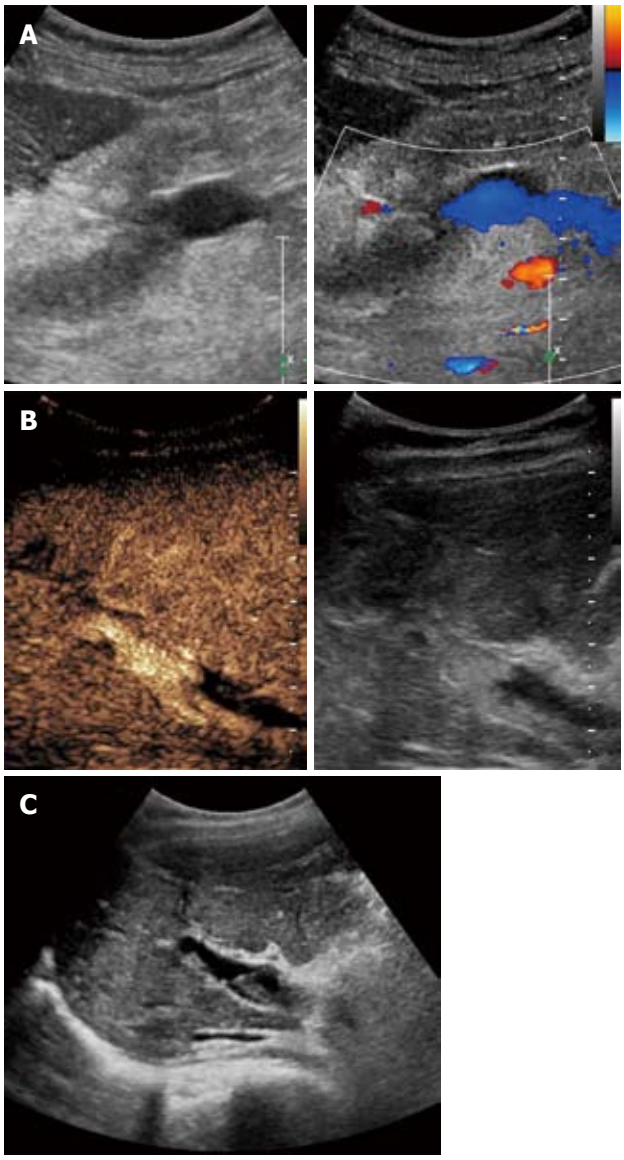


Figure 1 Portal vein thrombosis. A: Complete thrombosis of the portal vein trunk with patent splenic vein (B-Mode and Color Doppler imaging); B: Partial thrombosis of portal vein right branch at B-Mode and contrast-enhanced ultrasound imaging; C: Partial thrombosis of portal vein right branch at B-Mode imaging.

Table 1 Risk factors most frequently involved in portal vein thrombosis. Development in cirrhotic patients, according to the Virchow triad, with the addition of vascular invasion

Circulatory stasis	Procoagulant status	Vascular injury	Vascular invasion
Cirrhosis	Inherited:	Trauma	Hepatocellular carcinoma
	Factor V Leiden mutation	Surgery (e.g., splenectomy colectomy, gastrectomy cholecystectomy	Cholangiocarcinoma
	Factor Ⅱ (prothrombin) mutation	Liver transplantation	
	Protein C deficiency	Invasive procedures (TIPS placement, fine needle aspiration, radiofrequency)	
	Protein S deficiency antithrombin deficiency	Chemical irritation (alcoholization)	
	Acquired:	Infections	
	Myeloproliferative disorder antiphospholipid syndrome	Focal inflammatory lesions (e.g., diverticulitis, appendicitis, pancreatitis, duodenal ulcer, cholecystitis, tuberculous)	
	Hyperhomocysteinemia		
	Malignancy (any abdominal organ)		
	Pregnancy/puerperium		
	Oral contraceptives		
	Sepsis		

TIPS: Transjugular intrahepatic portosystemic shunt.

increase of factor Ⅷ and D-dimer was observed in those with advanced liver cirrhosis. High levels of factor Ⅷ have already been reported in cirrhotic patients and it is now well known that they represent an intrinsic feature of the disease (plasmatic levels of factor Ⅷ beyond the 66th percentile - 129 IU/dL - are reported to have a 6-fold greater risk of PVT development[20]), while the increase in D-dimer is rather considered to be a non-specific finding[21]. However, the only conclusion the author could provide was that low values of D-dimer had high sensitivity and specificity in excluding PVT[22]. Among the other inherited pro-thrombotic disorders, prothrombin gene *G20210A* mutation seems to be the most frequent in cirrhotics with PVT[23-27], while the role of anticardiolipin antibodies is still a matter of concern, due to a high susceptibility to infections and to the presence of liver damage[28-33]. Recently, it has also been reported the presence of the *JAK2 V617F* gene mutation in about 17%-35% of PVT patients without a known underlying disease[34-36]; however, even if it could be a marker of a myeloproliferative disorder before it becomes clinically evident, the prevalence among cirrhotic PVT patients is actually unknown.

PVT DEVELOPMENT AND THE PROGRESSION OF LIVER FIBROSIS/CIRRHOSIS

Is there a link between the progression of liver disease throughout the development of liver cirrhosis and portal vein thrombosis? The answer may be "yes". Different factors appear to be involved in chronic liver injury; among them, the coagulation system appears to be particularly involved. Animal models of cirrhosis confirmed that mice carrying the factor V Leiden mutation exhibit more severe fibrosis than wild-type littermates[37,38]. In other clinical studies, factor V Leiden mutation was reported to be associated with a 3.28-fold increased risk of rapid fibrosis progression in patients with hepatitis C[39]. A similar tendency has been demonstrated in patients with protein C deficiency, increased factor Ⅷ expression and hyperhomocysteinemia[40]. The main link between hypercoagulability and fibrosis progression is still being investigated, but it is more likely due to a reaction to liver tissue ischemic damage. It has been postulated that micro-infarcts resulting from thrombosis of the hepatic and portal vein branches may cause ischemia and cell death, with consequent parenchymal collapse manifesting with areas of parenchymal extinction[41,42]. These areas are then replaced by fibrous tissue producing cirrhosis[37]. Liver injury is able to activate the perisinusoidal hepatic stellate cells, which can transdifferentiate into myofibroblasts, showing pro-inflammatory and pro-fibrogenic activity[38,43]. A similar mechanism of intrahepatic thrombosis of portal vein branches has been recognized at the origin of nodular regenerative hyperplasia development, which could be confused with liver cirrhosis[44].

Indeed, the t-PA-plasmin system is involved in activation of transforming growth factor-beta (TGF-β), a pro-fibrogenic cytokine, which is essential in the pathogenesis of liver cirrhosis. Wanless *et al*[41,42] demonstrated that microthrombi are frequently present within hepatic vessels of patients with liver cirrhosis and other studies showed a correlation between inherited thrombophilic conditions and severity and progression of liver disease. Moreover, hepatitis C virus infected haemophilic patients seem to have a slower disease progression, supporting the hypothesis that a hypercoagulant state may heavily worsen the natural history of liver disease[45].

Even if the exact mechanism is still unknown, a primary role of parenchymal extinction and of stellate cells activation in triggering this process has been postulated. Indeed, vascular microthrombosis secondary to necroinflammation, may cause liver ischemia and infarction, with loss of hepatic tissue (parenchymal extinction) which is replaced by fibrotic tissue. Cirrhosis will therefore result from the confluence of these microfibrotic areas. Alter-

natively, thrombin may induce fibrogenesis by itself, both activating specific receptors on stellate cells, or indirectly, stimulating release of platelet-derived growth factors by platelets and promoting post-translational activation of TGF-β[37,39,40,46]. The first of these is a direct mechanism; G-protein coupled protease activated receptor (PAR)-1, PAR-3 and PAR-4 are associated to thrombin signaling[47-50]. Thrombin exerts a chemotactic effect on monocytes and a mitogenic stimulus for smooth muscle cells, fibroblasts and hepatic stellate cells[51], which express members of the PAR family *via* PAR-1[52-54]. Therefore both an increased generation of thrombin, due to the activation of the coagulation system, and the lack of an adequate inhibition of thrombin generation, as in patients carrying the factor V Leiden mutation or PC deficiency, may contribute to liver fibrogenesis. Elevated levels of factor Ⅷ may also activate PAR-1 signaling[55].

The role of FXa/PAR-2 signaling in hepatic fibrosis is less well characterized, however, there is now evidence for its role in several fibrotic diseases through fibroblast activation including α-smooth muscle actin production, monocyte chemoattractant protein-1 and interleukin-6 secretion, and TGF-β expression[51]. Moreover, FXa signaling *via* PAR-1 remains the most important although this may vary in different tissues[56].

In conclusion, stellate cells activation and parenchymal extinction play a pivotal role in determining liver fibrosis evolution, with a recognized trigger in the coagulation system[33]. In this perspective, PVT development should not only be considered as a matter of impaired blood flow or pro-coagulation tendency, but also as the overt manifestation of the liver fibrosing process.

HOW MUCH DOES PVT IMPACT ON THE NATURAL HISTORY OF LIVER CIRRHOSIS?

Impact on complications

In the past years, cirrhotic patients developing PVT where considered at high risk of mortality for gastrointestinal bleeding, especially in case of complete occlusion. The risk of variceal bleeding is nearly 80-120 times higher in cirrhotics with PVT than in those without liver disease (19%-40%)[17,19,57]. Therefore an episode of unexpected variceal hemorrhage can be linked with a high probability to acute PVT development, as well as refractory ascites in a patient previously responsive to the diuretic treatment[58]. In addition, when the thrombus extends to the superior mesenteric vein, the risk of intestinal infarction is high[59].

PVT has been found to be an independent predictor for failure to control active variceal bleeding and prevent variceal re-bleeding, irrespective of the use of endoscopic hemostasis or surgical shunting as treatment modalities[60,61]. There are some doubts about a supposed favoring role of the endoscopic therapy of esophageal varices in PVT development; probably, varices represent by themselves a marker of portal hypertension, which might be a risk factor for PVT independently of the procedure[59].

Recent data highlight that the prevention of PVT development is a useful tool to avoid the occurrence of decompensation in cirrhotic patients. Zecchini *et al*[62] randomized 51 cirrhotics with advanced liver disease (Child B7-C10) to receive enoxaparin 4000 IU/d (26 patients) or placebo (25 patients) for 12 mo; PVT occurred in 5 patients in the placebo group (2 complete, 3 partial) and in none in the treatment group. Treated patients experienced fewer episodes of decompensation.

Impact on survival

The natural history of cirrhosis in patients with PVT is largely unknown. In the past, patients with PVT were considered to be at increased risk of mortality related to bleeding complications, but the improvement in gastrointestinal bleeding management has changed this perspective[23].

Ferreira *et al*[63] reported data about prognosis of non-tumoral PVT in 40 consecutive cirrhotic patents (median follow-up 6.5 mo). Fifty-eight percent of patients survived at the end of follow-up and PVT was associated with increased risk of mortality at 3 years (but not at 1 year) compared to controls, and this effect was evident only in patients with Child Pugh score lower than 10 (Child Pugh A and B patients).

Englesbe *et al*[64] recently performed a retrospective study to evaluate the survival of cirrhotic PVT patients including a small sample of 148 cirrhotic patients presenting with occlusive PVT followed-up at the University of Michigan during a large period (1995-2007). In this study, which has the limitation of retrospective studies, only patients undergoing pre-transplant screening were included, and there was no certain data regarding the presence of pro-thrombotic disorders, administration of anticoagulants and thrombosis grading. The authors reported 54.7% of deaths in patients with PVT versus 37.2% in patients without PVT. Interestingly, from the time of the initial evaluation, PVT patients had a risk of mortality similar to that of patients with a MELD score of 26, even if their MELD score at the time of LT evaluation was of 12. This risk increased over the time, reaching that of patients who presented for listing with a MELD score of 31. PVT was also showed to influence short-term survival after LT too. Differently from other previous studies, this by Englesbe *et al*[64] took into account MELD score.

On this basis, Englesbe *et al*[64] postulated that, if patients with PVT have significantly worse waiting list survival and an increased mortality, they should consequently have an augmented access to LT. However, the most used allocation system, based on the classification of waiting list patients according to the MELD score, may not represent the real priority of PVT patients as well, since they could maintain a good liver function for long times. Moreover, the assignation of MELD extra points, such as in case of hepatocellular carcinoma, has not yet been introduced in PVT patients. Therefore, Englesbe *et al*[64]

tried to assess the real LT benefit for patients with PVT analyzing data on adult liver transplant candidates waitlisted between September 2001 and December 2007 from the Scientific Registry of Transplant Recipients. Candidates with PVT had similar survival rates on the waiting list compared to those without PVT, but they had a significantly inferior post-LT survival. Therefore, even if a low MELD score may reflect better clinical conditions for liver candidates, with obvious benefits in terms of peri- and post-LT outcomes, especially in presence of PVT that seems by itself to complicate the LT procedure, it seems disadvantageous to allow an early access to LT for cirrhotic patients with PVT. Specifically, the threshold for transplant benefit among patients without PVT was MELD score > 11 compared to MELD score > 13 for patients with PVT. The authors concluded that clinicians should carefully consider the risks of LT in clinically stable patients with PVT. Another recent interesting but small study confirmed this evidence[65]. In this study PVT was related to a decreased post-LT survival only in patients with a low MELD score, while no difference in outcome between PVT and non-PVT patients were found for MELD scores > 15. Notably, there was no difference in PVT extension between the groups with high and low MELD scores. Indeed, the impact of PVT on patients' survival seems to be dependent by its extension; Doenecke *et al*[65] reported 90% 1 year post-LT survival for PVT grade 1/2 patients, compared to 57% for PVT grade 3/4. Except for a slightly higher transfusion requirement for PVT patients, intraoperative parameters as well as the duration of in-hospital stay and the incidence of post-operative liver dysfunction or failure did not differ significantly between high and low MELD PVT groups. In the study by Englesbe *et al*[12] the presence of occlusive PVT at the time of LT was associated with an increased 30 d risk of mortality. Therefore, the increased risk of post-LT mortality in PVT patients seems not only a matter of MELD score[65].

Taking together all these considerations, the outcome of PVT patients undergoing LT still seems to be controversial, almost similar to that of non-PVT patients or rather worse. Even if some authors conclude that no increased risk of mortality was observed for PVT patients while on waiting list and that there was no transplant benefit with an early LT, these considerations should be more cautious. Certainly, PVT grading could be a useful tool to identify those patients with a higher risk of mortality or complications. The observation that PVT patients are not at increased risk of mortality may be a too hazardous generalization of a more complex problem, and the prioritization of these patients should be evaluated on a single case basis.

CONCLUSION

Interesting perspectives may be opened by the connection between hypercoagulation, intrahepatic microthrombosis and progression of liver fibrosis. In particular, stellate cell activation and parenchymal extinction seem to play together a pivotal role in determining liver fibrosis evolution, with a recognized trigger in the coagulation system[33]. From this point of view, PVT development should not only be considered as a matter of impaired blood flow or pro-coagulation tendency, but also as the overt manifestation of liver fibrosing process evolution. Moreover, fibrogenesis itself could be controlled by targeting thrombin generation or its downstream signaling. It has been shown that administration of PAR-1 antagonist, thrombin inhibitor SSR182289, dipyridamole, rivaroxaban (a FXa inhibitor), vitamin K or low molecular weight heparin was able to reduce hepatic fibrosis in animal models[41,52,66-69]. Unfortunately, to date, data about the use of anticoagulants as anti-fibrotic therapy are scarce in humans but they are subject of many ongoing clinical trials.

As regards the second question of this review we can conclude that PVT represents an important event in the natural history of liver cirrhosis. We agree with Tripodi *et al*[55] that liver cirrhosis, slowing-down the physiologic flow of blood in the portal venous system, might favor PVT development but also that PVT development might have an impact on liver function by decreasing portal perfusion. This looping interaction confirms the strict connection between PVT and liver disease. First, PVT could not be merely considered as a problem to solve but, rather, as the alarm-bell that liver disease is evolving. In this perspective PVT prevention could be very important in order to control disease evolution and reduce episodes of decompensation as demonstrated by a recent study by Zecchini *et al*[62].

Second, the impact of PVT development on liver disease progression opens a new debate on the assignation of MELD extra points, such as in the case of hepatocellular carcinoma, in PVT patients on a waiting list for liver transplantation. This point of view is supported by the evidence that PVT cirrhotic patients have a high mortality risk compared to non-PVT ones from the time of evaluation for LT, which could be comparable to that of a MELD score as high as 26-31[64]. On the other hand, it seems that transplant benefit and the post-LT survival of PVT patients might not be advantageous in those with a low risk of liver disease-related mortality (low MELD scores)[12]. In our opinion, this problem should be seen from another perspective; the severity of liver disease may have a heavy impact on patients' survival despite the presence of PVT, which, in turn, should not represent a good reason to prioritize all patients on waiting list, especially those with a low MELD score. However, the presence of major complications, such as chronic hepatic encephalopathy, refractory ascites, recurrent variceal bleeding, with either major or minor bleeding episodes with scarce response to the endoscopic or medical therapy, could represent reasons for the prioritization on waiting list for patients with PVT. Probably, PVT extension should also be considered since it has been reported to impact on post-LT survival[65]. Even

if we are far away from the solution of this debate, we would like to remark that in those countries (e.g., Italy, United States) where the access to LT is determined by the risk of mortality on the waiting list, it is essential to determine how PVT could increase the risk of mortality of cirrhotics awaiting for LT. In this age characterized by organ shortage, liver graft loss should be avoided, thus maximizing the benefit of LT.

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ORIGINAL ARTICLE

Transrectal ultrasound and magnetic resonance imaging measurement of extramural tumor spread in rectal cancer

Søren R Rafaelsen, Chris Vagn-Hansen, Torben Sørensen, John Pløen, Anders Jakobsen

Søren R Rafaelsen, Chris Vagn-Hansen, Torben Sørensen, Department of Radiology, DCCG South, Institute of Regional Health Services Research, University of Southern Denmark, Vejle Hospital, Kabbeltoft 25, 7100 Vejle, Denmark
John Pløen, Anders Jakobsen, Department of Oncology, DCCG South, Institute of Regional Health Services Research, University of Southern Denmark, Vejle Hospital, Kabbeltoft 25, 7100 Vejle, Denmark
Author contributions: Rafaelsen SR, Vagn-Hansen C, Sørensen T, Pløen J and Jakobsen A were involved in the study conception, design, critically revising the article and approved the final version; Rafaelsen SR, Vagn-Hansen C and Sørensen T performed the imaging procedures; Rafaelsen SR analysed the data and wrote the manuscript.
Correspondence to: Søren R Rafaelsen, MD, Department of Radiology, DCCG South, Institute of Regional Health Services Research, University of Southern Denmark, Vejle Hospital, Kabbeltoft 25, 7100 Vejle,
Denmark. soeren.rafael.rafaelsen@slb.regionsyddanmark.dk
Telephone: +45-79-405336 Fax: +45-70-406873
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Abstract

AIM: To evaluate the agreement between transrectal ultrasound (TRUS) and magnetic resonance imaging (MRI) in classification of ⩾ T3 rectal tumors.

METHODS: From January 2010 to January 2012, 86 consecutive patients with ⩾ T3 tumors were included in this study. The mean age of the patients was 66.4 years (range: 26-91 years). The tumors were all ⩾ T3 on TRUS. The sub-classification was defined by the penetration of the rectal wall: a: 0 to 1 mm; b: 1-5 mm; c: 6-15; d: > 15 mm. Early tumors as ab (⩽ 5 mm) and advanced tumors as cd (> 5 mm). All patients underwent TRUS using a 6.5 MHz transrectal transducer. The MRI was performed with a 1.5 T Philips unit. The TRUS findings were blinded to the radiologist performing the interpretation of the MRI images and measuring the depth of extramural tumor spread.

RESULTS: TRUS found 51 patients to have an early ⩾ T3 tumors and 35 to have an advanced tumor, whereas MRI categorized 48 as early ⩾ T3 tumors and 38 as advanced tumors. No patients with tumors classified as advanced by TRUS were found to be early on MRI. The kappa value in classifying early versus advanced T3 rectal tumors was 0.93 (95% CI: 0.85-1.00). We found a kappa value of 0.74 (95% CI: 0.63-0.86) for the total sub-classification between the two methods. The mean maximal tumor outgrowth measured by TRUS, 5.5 mm ± 5.63 mm and on MRI, 6.3 mm ± 6.18 mm, P = 0.004. In 19 of the 86 patients the following CT scan or surgery revealed distant metastases; of the 51 patients in the ultrasound ab group three (5.9%) had metastases, whereas 16 (45.7%) of 35 in the cd group harbored distant metastases, P = 0.00002. The odds ratio of having distant metastases in the ultrasound cd group compared to the ab group was 13.5 (95% CI: 3.5-51.6), P = 0.00002. The mean maximal ultrasound measured outgrowth was 4.3 mm (95% CI: 3.2-5.5 mm) in patients without distant metastases, while the mean maximal outgrowth was 9.5 mm (95% CI: 6.2-12.8 mm) in the patients with metastases, P = 0.00004. Using the MRI classification three (6.3%) of 48 in the MRI ab group had distant metastases, while 16 (42.1%) of the 38 in the MRI cd group, P = 0.00004. The MRI odds ratio was 10.9 (95% CI: 2.9-41.4), P = 0.00008. The mean maximal MRI measured outgrowth was 4.9 mm (95% CI: 3.7-6.1 mm) in patients without distant metastases, while the mean maximal outgrowth was 11.5 mm (95% CI: 7.8-15.2 mm) in the patients with metastases, P = 0.000006.

CONCLUSION: There is good agreement between TRUS and MRI in the pretreatment sub-classification of ⩾ T3 tumors. Distant metastases are more frequent in the advanced group.

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Key words: Ultrasound; Magnetic resonance imaging; Rectal cancer; Tumor staging; Metastases

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INTRODUCTION

Depth of tumor spread beyond the rectal wall has been validated as an important prognostic indicator[1-3]. Tumors with 5 mm or less of extramural spread regardless of node status have an 85% cancer-specific survival rate compared with poorer-prognosis tumors with more than 5 mm spread, which have only a 54% 5-year cancer-specific survival rate[4]. The MERCURY study[5] showed that tumor spread can be estimated accurately with magnetic resonance imaging (MRI) in comparison with pathologic results. Studies have shown that there was good to very good agreement on measuring the maximal extramural tumor spread using MRI compared to histopathology[6,7]. The clinical guidelines for neoadjuvant therapy vary among countries.

The tumor extending 5 mm or more into perirectal fat is considered a high-risk disease[8]. Patients with a tumor infiltration depth more than 5 mm are at some institutions offered preoperative long-course chemoirradiation therapy. However, MRI cannot be used in all patients due to contraindications, pacemaker, claustrophobia, *etc*.

Transrectal ultrasound (TRUS) has been shown to be accurate in staging early rectal tumors[9-12] including accurate evaluation of penetration of the rectal wall[13-17]. The ability of TRUS to discriminate between early and advanced tumors has not been well documented. The aim of the present study was to compare the intermodality agreement of TRUS with MRI in measuring the depth of extramural tumor spread in rectal tumors, and to determine the incidence of distant metastases with regard to tumor invasion measured by TRUS.

MATERIALS AND METHODS

The study was approved by the local ethics committee and by the National Data Protection Agency (jr. nr. 2011-41-6988), according to national law.

From January 2010 to January 2012, 153 consecutive patients with suspected rectal carcinomas were referred to the Department of Radiology for local staging. The patients were retrieved in the Radiology Information System (Kodak Carestream RIS 2008, Eastman Kodak Company, NY, United States). Fourteen patients having neither TRUS nor MRI were excluded as well as 11 patients with other cancers. The reasons for exclusion are listed in Table 1. A total of 86 patients with sonographical ⩾ uT3 tumors were included in this study, five of these were uT4. The mean age of the patients was 66.4 years (range: 26-91 years). Forty-five of the 86 patients were treated with radio-chemotherapy. The diagnosis of rectal carcinoma was confirmed in all patients by histopathological examination of the biopsy material. The sub-classification of ⩾ T3 was defined as: a with a penetration of the rectal wall from 0 to 1 mm, b: 1-5 mm, c: 6-15 mm, d: > 15 mm. Early ⩾ T3 tumors as ab (⩽ 5 mm penetration) and advanced tumors as cd (> 5 mm penetration).

All patients underwent TRUS using a 6.5 MHz forward looking transrectal transducer (EC9-4) connected to a Siemens S 2000 ultrasound machine (Issaquah, Washington, United States). The transducer was used to facilitate visualization of stenotic tumors. Patients received a cleansing enema prior to their examination and all procedures were carried out with the patient in the left lateral position. All observers who performing and interpreting the TRUS were specialists in radiology with more than 10 years' experience in TRUS and all had participated in a special training course. The level above the anal verge and location of the tumor were not blinded to the observers. The extent of wall invasion was assessed according to uT criteria as described by Hildebrandt *et al*[18]. In all ⩾ T3 patients in the study population the maximal depth of extramural tumor spread was prospectively measured in mm and the ultrasound images were stored in the radiological electronic picture archive computer system (PACS).

The MRI scan was carried out using an up-graded 60 cm bore Achiva 1.5 Tesla MRI unit with a 5-element wrap-around surface coil over the pelvis (Philips, Eindhoven, The Netherlands). After localizer scans, fast T2-weighted (w) spin-echo sequences were obtained. The scan included high-resolution 3 mm axial slices at a 90-degree angle to the tumor. The axial scans were prepared by the MR radiographer with assistance from the radiologists to ensure perpendicular images. No contrast enhancement was used. Each patient underwent the TRUS and MRI examinations on the same day. The MRI images were interpreted and reported by two experienced radiologist after the TRUS examination in the daily clinical setting. The depth of extramural tumor spread was measured blindly to the TRUS findings in this study, with special focus on the axial MRI images. All images were evaluated using an Impax PACS workstation (Agfa, Mortsel, Belgium) with two Coronis monitors (1600 1200 pixels; Megapixels Diagnostic Display System; Barco, Kortijk, Belgium). Presence or absence of any distant metastasis at subsequent CT and surgery was recorded.

Table 1 Transrectal ultrasound and magnetic resonance imaging were requested in 153 patients with rectal tumors detected by endoscopy

Patients with suspected rectal cancer admitted to local staging using TRUS and MRI	*n*
Excluded:	
No MRI: pacemaker: 3; claustrophobia: 1	4
No TRUS: poor cleansing: 4; pain: 2; refused: 2	8
Other cancers excluded: anal cancer: 7; carcinoid: 2; GIST: 1; prostate cancer: 1	11
Adenocarcinomas excluded: duplex tumour: 1; stent: 1; sigmoid tumour: 5	7
< uT3: uT1: 9; uT2: 18	27
uT3 with insufficient documentation of outgrowth measurement	4
uT4 with insufficient documentation of outgrowth measurement	6
Total study group	86

Of these, 67 were patients excluded from the study. TRUS: Transrectal ultrasound; MRI: Magnetic resonance imaging; GIST: Gastrointestinal stromal tumor.

Table 2 Extramural invasion measured by transrectal ultrasound and magnetic resonance imaging

	MRI-a	MRI-b	MRI-c	MRI-d
TRUS-a	10	6		
TRUS-b	1	31	3	
TRUS-c			25	3
TRUS-d			2	5

TRUS: Transrectal ultrasound; MRI: Magnetic resonance imaging. The sub-classification of ⩾ T3 was defined as: a: 0 to 1 mm; b: 1-5 mm; c: 6-15 mm; d: > 15 mm.

Statistical analysis

Statistical analysis were performed using Number Cruncher Statistical Systems (NCSS, Kaysville, Utah, United States). Intermodality agreements were assessed with Cohen's kappa statistics. Kappa values were interpreted the following way: absence of agreement 0, slight agreement 0.20, fair agreement 0.21-0.40, moderate agreement 0.41-0.60, substantial agreement 0.61-0.80, and almost perfect agreement 0.81-1.0 as proposed by Landis *et al*[19]. Confidence limits were set at 95 percent. Descriptive statistics were used. Means between TRUS and MRI were analyzed using Paired *t* test. Comparison of proportions was performed by χ^2 test or, when appropriate, Fisher's exact test. *P* values less than 0.05 were considered significant.

RESULTS

TRUS found 51 patients to have an early ⩾ T3 tumor and 35 to have an advanced tumor, whereas MRI categorized 48 as early ⩾ T3 tumors and 38 as advanced tumors (Table 2). Three patients had tumors characterized as early by TRUS (uT3b), but advanced by MRI; however the extent of outgrowth was only 6 mm when measured by MRI. No patients with tumors classified as advanced by TRUS were found to be early on MRI. The unweighted and linear weighted kappa values in classifying early versus advanced ⩾ T3 rectal tumors were both 0.93 (95% CI: 0.85-1.00).

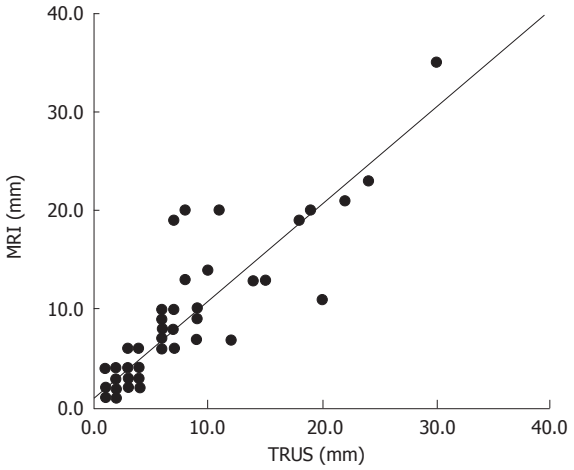


Figure 1 Plot graph showing agreement on depth of extramural tumor spread in mm. The x-axis represents the distance measured by transrectal ultrasound (TRUS) and the magnetic resonance imaging (MRI) results are shown on the y-axis. Correlation coefficient = 0.90.

The results of the subgroup classification are shown in Table 2. TRUS sub-classified 16 patients as uT3a, while MRI categorized six of these as ⩾ T3b. The actual outgrowth measure by MRI was 2 mm in five and 4 mm in one of these patients. One MRI a sub-stage was b sub-stage using TRUS, the difference was 1 mm in this patient.

Three MRI d-group patients were only c-group on TRUS. Two patients had a difference of 12 mm and one with 9 mm (Figure 1 and Table 2). Two were in the d-group on TRUS but on MRI the same patients were in the c-group; the difference in outgrowth was 9 and 2 mm, respectively. We found a kappa value of 0.74 (95% CI: 0.63-0.86) for the total sub-classification between the two methods (Figures 2 and 3).

Figure 1 shows plots of the data from TRUS versus MRI, with respect to maximum extramural tumor spread. Multiple values occurring at a specific point were more common where the tumor outgrowth was limited.

The overall mean maximal tumor outgrowth measured on TRUS, 5.5 mm ± 5.63 mm and on MRI, 6.3 mm ± 6.18 mm, $P = 0.004$. The highest value in the same patient was 30 mm on TRUS and 35 mm measured by MRI.

In 19 of the 86 patients the following CT scan or surgery revealed distant metastases; of the 51 patients in the ultrasound ab group three (5.9%) had metastases, whereas 16 (45.7%) of 35 in the cd group harbored distant metastases, $P = 0.00002$. The odds ratio was 13.5 (95% CI: 3.5-51.6), $P = 0.00002$. The mean maximal ultrasound measured outgrowth was 4.3 mm (95% CI: 3.2-5.5 mm) in patients without distant metastases, while the mean maximal outgrowth was 9.5 mm (95% CI: 6.2-12.8 mm) in the patients with metastases, $P = 0.00004$. Using the MRI classification three (6.3%) of 48 in the

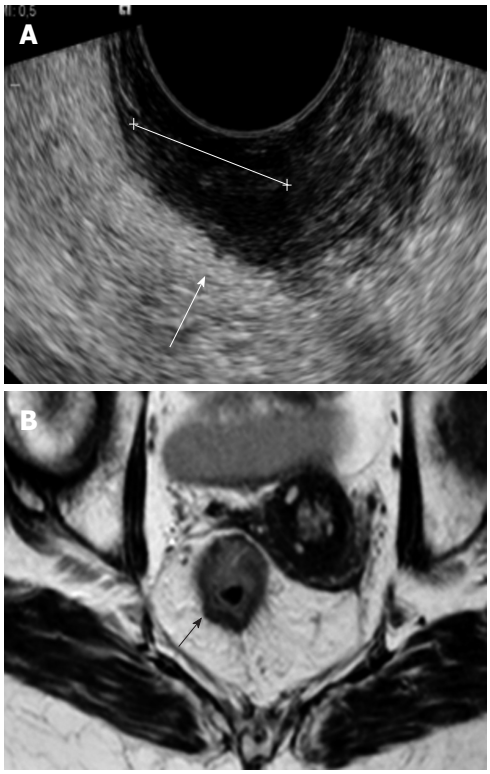


Figure 2 Early T3 rectal tumor on both transrectal ultrasound (A) and axial magnetic resonance imaging image (B). The maximal outgrowth (arrow) was 2 mm using both methods.

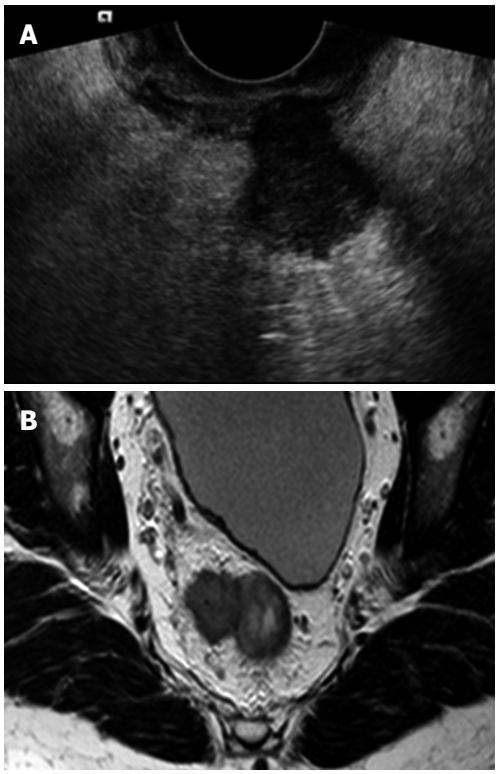


Figure 3 Transrectal ultrasound (A) and axial magnetic resonance imaging images (B) of a patient showing an advanced T3 rectal tumor. The maximal outgrowth on the right side measures 20 mm equally on transrectal ultrasound and axial magnetic resonance imaging.

MRI ab group had distant metastases, while 16 (42.1%) of the 38 in the MRI cd group, $P = 0.00004$. The MRI odds ratio was 10.9 (95% CI: 2.9-41.4), $P = 0.00008$. The mean maximal MRI measured outgrowth was 4.9 mm (95% CI: 3.7-6.1 mm) in patients without distant metastases, while the mean maximal outgrowth was 11.5 mm (95% CI: 7.8-15.2 mm) in the patients with metastases, $P = 0.000006$.

DISCUSSION

This first study on the correlation between TRUS and MRI in discrimination of early *vs* advanced rectal cancer might have clinical implications concerning patients with contraindications to MRI. The results indicate that these patients can be evaluated using TRUS to determine whether the patient has an early or advanced rectal cancer. Patients with claustrophobia, implanted cardiac prostheses, metallic cardiovascular electronic devices, intravascular stents and filters, cochlear implants, bullets, etc. are either not suitable for MRI or there is a serious risk involved in having an MRI performed[20]. Patient movements during the MRI scan are also associated with reduced image quality[21].

Patients with tumors confined to the rectal wall or early T3 tumors can be treated with surgery alone unless suspicious lymph nodes are detected, whereas tumors with advanced mesorectal involvement may benefit from preoperative treatment[22-24]. According to our results three patients were classified as T3b using TRUS, but as T3c using MRI. These three patients would not have been treated with radiochemotherapy using this setting if MRI was contraindicated. However, the invasion in the mesorectal fat was only 6 mm in all three MRI advanced patients.

The discrepancies in the sub-group staging using TRUS and MRI were only minor distances, except for a difference up to a 12 mm outgrowth in a patient with an advanced tumor on both modalities. In the patient with the most extensive outgrowth the measurement varied from 30 to 35 mm and was categorized equally as d-group with the two methods. The resolution of a 6.5 MHz transrectal tranducer is high and approximately the same or higher as the resolution for thin slice axial T2 weighted MRI, ranging about 0.3-0.4 mm. Thus, variances in resolution cannot explain this difference between large tumors. The reason could be measurements at different positions. TRUS can have difficulties in imaging the total volume of large tumors and perhaps this result in underestimation. The presence of a large tumor is significantly related to a poorer performance of TRUS[25]. This can in part explain why TRUS more often underestimated the tumors in the subclassification compared to MRI, than the vice versa (Table 2).

The anal sphincter was involved in three patients, seminal vesicles in a male patient and vagina in a female patient. A recent Japanese pathological study found a 5-year survival of 72.4% *vs* 30.0% in patients with early *vs* advanced T3/T4 tumors[26]. The depth of tumor

invasion beyond the rectal wall is considered to be an important prognostic factor for patients with T3 and T4 rectal carcinoma. The patients classified as advanced had an odds ratio of 13.5 of having distant metastases in the advanced group, although both uT1 and uT2 tumors were excluded. A new MRI study reported that patients with more than 5 mm tumor outgrowth have a higher risk of harboring distant metastases with an odds ratio of six and these patients may undergo more intensive preoperative staging including FDG-PET/CT and liver MRI and be considered for chemotherapy[27]. Another study found a higher incidence of lung metastases with higher T stage[28].

The main strength of the blinded study is that we compared state of the art TRUS with MRI using experienced readers. The ultrasound measurements were performed in a prospective clinical setting but analysed retrospectively. We did not have any lack of thin-slice or high-resolution T2-weighted MRI images perpendicular to the tumor resulting in satisfactory image quality. We did not find any different correlation between TRUS and MRI regarding the TRUS observers. An inter-observer variation study of infiltration depth of ≥ T3 tumors using TRUS will require a 3-dimensional stored image block of the ultrasound data which could be blindly re-evaluated. It was not possible in our clinical setting; an inter-observer study on plane two-dimensional images would introduce an operator bias.

Only a few previous studies have focused on the aspect of tumor outgrowth using ultrasound. Harewood *et al*[29] used TRUS in 14 patients with minimally invasive T3 and 28 patients with advanced T3 disease. They used 2 mm invasion as a cut-off. Digital images were obtained during each TRUS procedure, at least 10 images per patient, illustrating the deepest extent of tumor involvement. These images were reviewed by two observers and a kappa value of 0.62 was reported. The mean depth of invasion beyond the rectal wall was 3.9 mm *vs* 5.5 mm in the present study. The mean outgrowth was 9.5 mm in the metastatic group.

A recent study by Jürgensen *et al*[30] found 16 patients with minimal invasive uT3 tumors and 24 with advanced uT3 cancers. The depth of infiltration beyond the muscularis propria was determined retrospectively by evaluation of paper prints and reports. Six patients could not be classified retrospectively. This discrimination between minimally invasive and advanced cancers correlated well to the pathological stage. In our study we also found a good intermodality agreement between TRUS and MRI, if the cut off between early and advanced ≥ T3 tumors was less than 5 mm.

The patient population of the present study was uniform, containing all patients with advanced rectal cancer, for determining intermodality agreement. In the study period there was no screening programme running for colorectal cancer, favouring a relatively high proportion of advanced tumors. The staging of the stenotic tumors has been accomplished by the use of a forward looking transrectal transducers[31,32] as in the present study. Side-viewing transducers including rotating transducers do not have this possibility of adequate visualising stenotic tumors[33].

The retrospective setting allowed us to evaluate a large number of MRI scans within a relatively short time period, but may invite learning bias of the MRI findings. Another limitation is the use of MRI as reference and not the histopathology of the surgical specimens. The latter was not possible since the majority of the patients underwent preoperative radiochemotherapy. This may explain that our mean invasion depth beyond the rectal wall was more pronounced than in the study by Harewood *et al*[29].

Another drawback of this retrospective study is the exclusion of a few patients with missing values of the precise extent of the tumor outgrowth. Four of the excluded uT3 patients were from the first part of the study period and during vacations, but there was agreement on the T-stage between TRUS and MRI. Finally, TRUS cannot detect the mesorectal fascia as with MRI[34,35], and consequently, MRI is used as a first choice staging modality.

In conclusion, there was very good intermodality agreement between TRUS and MRI in the pretreatment in classification of tumors as early and advanced ≥ T3. Patients with more than 5 mm outgrowth measured by ultrasound have an increased risk of harboring distant metastases. TRUS can be used to predict advanced rectal cancers and thus be helpful if chemoradiation is considered in patients with contraindications to MRI.

COMMENTS

Background

Magnetic resonance imaging (MRI) is widely used in preoperative staging of rectal cancer. Patients with claustrophobia cannot benefit from this method. The depth of tumor outgrowth is an important prognostic factor.

Research frontiers

MRI has recently shown to be accurate in measuring tumor outgrowth and the MRI measured maximal depth of outgrowth seems to be related to presence of distant metastases.

Innovations and breakthroughs

Previously it has been shown that MRI measured extratumoral outgrowth is related to distant metastases. In the present study the authors have demonstrated an almost perfect agreement between MRI and transrectal ultrasound (TRUS) in categorizing early and advanced tumors.

Applications

TRUS can be used to discriminate between early *vs* advanced rectal cancer, and thus be helpful if chemoradiation is considered in patients with contraindications to MRI.

Peer review

Transrectal ultrasound and MRI measurement of depth of extramural tumor spread in rectal tumors Rafaelsen. In this study the authors investigated the depth of tumor spread beyond the rectal wall using MRI and ultrasound (US) techniques in patients with suspected rectal carcinomas. They correlated the data between MRI and US and to metastasis of the cancer. Extension of the tumor beyond the rectal wall was subclassified in units of millimeters. Early tumors were considered to have ≤ 5 mm penetration while advanced tumors were defined as those with > 5 mm penetration. Three patients had tumors characterized as early by US but advanced by MRI, where in the three cases the outgrowth measured by MRI was 6 mm. No patients with tumors classified as advanced by US were classified as early type using MRI. Overall this is an interesting and well done study.

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ORIGINAL ARTICLE

Effect of psychological stress on gastric motility assessed by electrical bio-impedance

María Raquel Huerta-Franco, Miguel Vargas-Luna, Juana Berenice Montes-Frausto, Ismael Morales-Mata, Lorena Ramirez-Padilla

María Raquel Huerta-Franco, Ismael Morales-Mata, Lorena Ramirez-Padilla, Department of Applied Sciences to Work, Health Science Division, University of Guanajuato, 37320 Leon, Guanajuato, Mexico
Miguel Vargas-Luna, Juana Berenice Montes-Frausto, Department of Physical Engineering, Science and Engineering Division, University of Guanajuato, 37150 Leon, Guanajuato, Mexico
Author contributions: Huerta-Franco MR and Vargas-Luna M are experts on the use of bio-impedance for gastric evaluation, designed and conducted the study and wrote the manuscript; Morales-Mata I performed data acquisition and instrumentation review; Montes-Frausto JB contributed to data collection, reviewed the literature and analytical input; and Ramirez-Padilla L contributed to the psychological data collection and performed the psychological tests.
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Correspondence to: Maria Raquel Huerta-Franco, PhD, Department of Applied Sciences to Work, Health Science Division, University of Guanajuato, Aquiles Serdan No. 924, Colonia Obregon, 37320 Leon, Guanajuato, Mexico. mrhuertafranco@ugto.mx
Telephone: +52-477-2569688 Fax: +52-477-7885100
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Abstract

AIM: To evaluate gastric motility using electrical bio-impedance (EBI) and gastric changes as a result of stress induced by psychological tests.

METHODS: A group of 57 healthy women, aged 40-60 years, was recruited, and a clinical history and physical examination were performed. The women were free from severe anxiety, chronic or acute stress, severe depression, mental diseases and conditions that affect gastric activity. The women were evaluated under fasting conditions, and using a four-electrode configuration, the gastric signals were obtained through a BIOPAC MP-150 system. The volunteers were evaluated using the following paradigm: basal state, recording during the Stroop Test, intermediate resting period, recording during the Raven Test, and a final resting period. We analyzed the relative areas of the frequency spectrum: A1 (1-2 cpm), A2 (2-4 cpm), A3 (4-8 cpm), and A4 (8-12 cpm), as well as the median of area A2 + A3. The data were analyzed by an autoregressive method using a Butterworth filter with MatLab and Origin. Analysis of variance (ANOVA) and Friedman ANOVA (for nonparametric variables) were performed; in addition, pairs of groups were compared using the *T* dependent and Wilcoxon *T* tests.

RESULTS: The results of the main values of area A2 were not significantly different comparing the five steps of the experimental paradigm. Nevertheless, there was a tendency of this A2 region to decrease during the stress tests, with recuperation at the final resting step. When an extended gastric region was considered (1-4 cpm), significant differences with the psychological stress tests were present ($F = 3.85$, $P = 0.005$). The A3 region also showed significant changes when the stress psychological tests were administered ($F = 7.25$, $P < 0.001$). These differences were influenced by the changes in the adjacent gastric region of A2. The parameter that we proposed in previous studies for the evaluation of gastric motility by electrical bio-impedance (EBI) was the median of the area under the region from 2 to 8 cpm (A2 + A3). The mean values of these frequencies (median of the A2 + A3 area) with the stress test showed significant changes ($F = 5.5$, $P < 0.001$). The results of the Wilcoxon *T* test for the A4 area parameter, which is influenced by the breathing response, changed significantly during the Raven stress test ($P < 0.05$).

CONCLUSION: We confirm that the gastric response to acute psychological stress can be evaluated by short-term EBI.

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Key words: Psychological stress; Bio-impedance; Gastric motility

Peer reviewers: Ming Li, Associate Professor, Tulane University Health Sciences Center, 1430 Tulane Ave Sl-83, New Orleans, LA 70112, United States; Guang-Yin Xu, MD, PhD, Assistant Professor, Division of Gastroenterology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77555-0655, United States; Cesare Tosetti, MD, Department of Primary Care, Health Care Agency of Bologna, Via Rosselli 21, 40046 Porretta Terme (BO), Italy

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INTRODUCTION

The relationship between emotions and gastric motility has been documented since the beginning of the last century by Cabanis and Beaumont and thereafter by Pavlov and Canon, who were the pioneers in determining the gastric response after an emotional stimulus in animal models[1-3]. In 1934, Hall described the autonomic response of rats exposed to unfamiliar events[1,4]. The effects of stress on the gastrointestinal (GI) tract have been reported since the first studies on the general adaptation syndrome by Selye[5] in 1936, who confirmed the physical, physiological and psychological responses of a living organism due to stress. In healthy humans, anger, fear, labyrinthine stimulation, painful stimuli, preoperative anxiety and intense exercise are some of the causes of slow gastric emptying[1,6,7]. The inhibition of gastric emptying and stimulation of colonic motor function are the most commonly encountered patterns induced by various stressors[1].

In the study of psychological stress as a risk factor for human health, there are few scientific reports in relation to the GI system, and most of the studies in humans have focused on the cardiovascular system because there is a clinical association of psychological and physical stress with a high risk for stroke and cardiovascular events, as well as with increased morbidity and mortality of the population. However, the evaluation of gastric function, particularly gastric motility and emptying, during psychological stress has not been given sufficient scientific attention despite the high prevalence of gastric problems due to psychological stress, such as gastritis, abdominal pain and gastroenteritis[8]. The alterations in the GI motility pattern by various stressors have been documented, although scarcely, with the development of quantitative techniques to monitor GI motility and transit in experimental animals and humans[9,10]. The gold standard technique accepted for gastric activity evaluation is scintigraphy, although this technique measures gastric emptying as the main consequence of gastric motility[11]; however, this technique is invasive. For the direct assessment of gastric motility, there are other invasive methods, such as manometry, which uses a probe placed at a specific point in the GI tract to record directly the frequency and amplitude of the GI movements[12].

Electrogastrography (EGG) is considered an invasive procedure when placing the electrodes in the GI cavity to record the myoelectrical activity of a specific inner surface of the tract[13,14]. However, EGG is a noninvasive technique in which the electrodes are placed on the skin. Another similar technique is the use of electrical bio-impedance (EBI), which also uses cutaneous electrodes in the gastric region[15-17]. In these last two techniques, the main challenge is the interpretation of the signal due to the overlapping information from different regions in the gastric system, particularly with respect to lung region movements in the case of EBI; in both cases, different motility frequencies are involved[16,18]. In general terms, gastric clinical research requires a strong link between the accuracy and reliability of the method and the comfort of the patient[14]. Thus, from this point of view, noninvasive EGG and EBI assessments have advantages over other techniques[19]. Therefore, the use of short-term EBI can be a good tool to evaluate the gastric response to psychological stress.

Furthermore, the possibility of permanent monitoring at a low cost is the preamble for a feedback program to be used in stress treatment and control. Gastric signals are not frequently used for this purpose, although this physiological function is definitively affected by stress. Gastric waves occur at a rate of 3 cpm on average and up to 12 cpm in the intestinal region, therefore, a long recording period is likely needed to assess gastric motility accurately, which contrasts with the need for short, acute stress tests and hence the need for short-term gastric motility recordings. Our hypothesis is that by observing the relative changes of the entire GI activity in the window of frequencies from 1 to 8 cpm, gastric motility can be used to monitor the responses to acute stress that results from the application of psychological tests that last < 10 min, such as the Stroop[20,21] and Raven tests[22].

MATERIALS AND METHODS

Subjects

A group of 57 healthy women, aged 40-60 years (mean ± SD: 48.19 ± 5.98 years), was recruited after the protocol was approved by the Division of Health Sciences Campus Leon at the University of Guanajuato, Mexico. Written informed consent was obtained from all participants, and the study was conducted according to the guidelines outlined in the Declaration of Helsinki (World Health Organization, 1996)[23].

Clinical evaluation

Before starting the experiment, clinical assessments were

conducted for all participants. These assessments included the following: (1) general information, including name, age, sex, and occupation; (2) clinical history, with a clinical evaluation of GI-related diseases; (3) physiological data, anthropometric measurements, and body mass index; (4) lifestyle information, including exercise, sleep habits, and substance abuse (tobacco, alcohol, medication); and (5) additional information obtained by a physical examination. For the purpose of this study, this information was used to assure the internal validity of the study (homogeneity of the study group and the absence of health problems related to the GI tract). To participate in the study, the women had to have met the following criteria: 40-60 years of age, no prior GI disease, and no prior disease that may affect the GI system (diabetes, Parkinson's disease, amyloidosis, myotonic dystrophy, polymyositis, human immunodeficiency virus infection or cytomegalovirus infection). The women were required not to have been taking any treatment that could interfere with gastric activity, have any other endocrine disorder, or have had significant weight loss within 3 mo prior to the study. In addition to these physical criteria, the women had to be free from psychological problems, such as severe anxiety, chronic or acute stress, severe depression and mental disease, because these conditions could potentially affect gastric activity though endocrine and central nervous system modulation[1].

Gastric EBI recordings

Prior to the evaluation, each woman was asked to arrive under fasting conditions (more than 8 h). In addition, the women refrained from smoking, alcohol consumption, strenuous exercise and caffeine for 24 h before the evaluation. The women were evaluated while in the semi-Fowler supine position to enable them to observe a screen on the wall with the projection of the psychological test (Stroop and Raven tests). For this experiment, we used the four-electrode configuration. This configuration consisted of two electrodes placed on the abdomen and two on the back. One pair (one electrode placed on the back and the other placed on the anterior part) was used for the current injection, and the other pair was used for voltage recording. The first electrode was placed near the rib cage using the midline of the abdomen as a reference. The second electrode was placed approximately 5 cm from the first, 45 degrees down and left of the umbilicus. The back electrodes were placed at 2.5 cm off the spine at the level of both electrodes on the abdomen. The electrode configuration used in this study was similar to that used in previous studies, which has shown good results[24]. The gastric signals were obtained through a BIOPAC MP-150 system and the corresponding EBI module[24]. The rough data mainly show oscillations due to breathing and a slower oscillation due to gastric movements (Figure 1).

Paradigm of the stress test

The gastric function for all volunteers was evaluated before, during and after being subjected to two psychological tests that have been shown to produce psychological stress: the Stroop and Raven tests[20-22]. The Stroop Test consisted of a 3-min session during which words in color were projected upon a screen at the rate of one word per second. The participant was asked to say the color of the word. During the first 40 s, words that name several objects were projected on a screen, followed by 1 min of words that name colors. The written color sometimes corresponded to the color of the word and sometimes did not[20]. Finally, words that named several objects but not colors were shown[21]. The Raven Test is a psychological test to determine the intelligence quotient (IQ), which consists of solving sequences and completing figures or truncated shapes. This test was performed without a time limit because the purpose was not the evaluation of IQ but simply to induce a state of psychological stress in the participant[22].

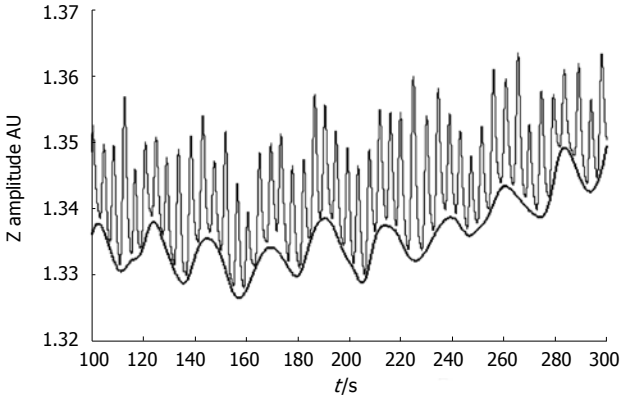


Figure 1 Raw data of the gastric bio-impedance signal showing mainly the breathing oscillations (fast oscillations) and the gastric contribution (2-4/min), which are shown by the dashed curve. AU: Arbitrary units.

The following paradigm was used in this study: (1) 10 min of basal gastric motility (BGM) recording by EBI (before the test); (2) EBI recording during the Stroop Test, lasting 3 min; (3) an intermediate resting period (IRP) of 3 min; (4) EBI recording during the Raven Test, lasting approximately 4 min; and (5) the final resting period (FRP), lasting 3 min.

Data analysis

In general, the parameter used to evaluate gastric motility is the position of the main peak in the region from 2 to 4 cpm of the frequency spectrum of gastric activity. In this study, we proposed alternative parameters when EBI was used[16] to examine the gastric motility changes due to psychological stress. We proposed the analysis of the relative area under the frequency domain curve in different regions of the spectrum as a relative measure of the gastric global activity in each frequency region. The frequency range was divided into four regions: the first (A1) included frequencies from 1 to 2 cpm and defined the low frequencies; the second (A2) included the frequencies from 2 to 4 cpm and defined the main frequency range of the gastric region activity according to

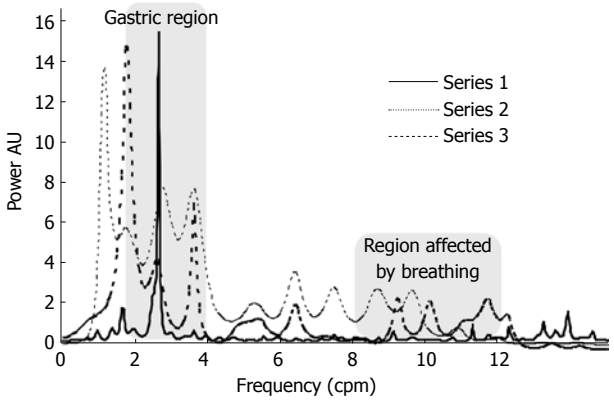


Figure 2 Typical frequency spectrum of the electrical bio-impedance gastric signal. The three curves show the typical changes during the tests (Stroop Test in this example): series 1: solid line, before the test; series 2: dotted line, during the test; series 3: dashed line after the test. AU: Arbitrary units.

the literature[1,7,9] (although some authors define a more restricted region for gastric motility as 2.5 to 3.75 cpm); the third (A3) included the frequencies from 4 to 8 cpm and contained the frequencies for the regions where bowel movements were important, which was mainly from the ileum (7-8 cpm)[1] or when tachygastria was present; and the fourth (A4) included the frequencies from 8 to 12 cpm and accounted for duodenum motility, breath frequency and tachygastria. The latter region, due to the strong contribution of the respiratory system, was not considered useful for gastric activity analysis because stress causes the breath frequency to change to higher values, diminishing the contribution of this area. A typical frequency spectrum and gastric changes occurring during the stress tests is shown in Figure 2.

We recorded the areas relative to the total area A_T from 1 to 12 cpm. In addition, the median of the area from 2 to 8 cpm (frequency for which the area under the spectrum was divided into two equal regions) was considered for analysis. This median changed when the relative areas of 2-4 cpm and/or 4-8 cpm changed, providing a good indication of the importance of the gastric motility compared with the adjacent frequency region. The advantage of these parameters was that small changes in the frequency spectrum, due to small variations in the data or data analysis, did not significantly affect these global parameters.

Statistical analysis

The data were analyzed by an autoregressive method using a Butterworth filter in the frequency range from 1 to 12/min (or cpm), i.e., 0.017-0.2 Hz. Particular attention was given to the region from 2 to 4/min (0.033-0.066 Hz) and from 4 to 8/min (0.066-0.132 Hz). In addition to this range, we analyzed the adjacent region, from 1 to 2 cpm, presented as the integrated region from 1 to 4 cpm, which is viewed as an extended gastric region. These analyses were performed using MatLab 6.5 and Origin 6.0 according to previous studies[16,24].

The comparison analysis within and between the experimental paradigm steps (BGM, Stroop Test, IRP, Raven Test, FRP) was performed using analysis of variance (ANOVA) and *post-hoc* analysis for variables with a normal distribution (A2, A1 + A2, A3, and the median under A2 + A3) and using the Friedman ANOVA for variables with a non-normal distribution (A4). Considering that the same women were evaluated before and after the psychological stress tests, we compared the dependent variables in the different experimental paradigm steps (BGM, Stroop Test, IRP, Raven Test, FRP) using the T dependent test on the normally distributed data (A1, A2, and A3 regions and the median of the area under the A2 + A3 region), and the T of the Wilcoxon test when non-normal distribution was found (A4 region). The statistical analyses were performed using STATISTICA 7 (Stat-Soft, Inc. Tulsa, OK, United States). We considered a P value $\leqslant 0.05$ to be significant.

RESULTS

The results of the gastric activity A2 area, indicated by the main range from 2 to 4 cpm, are presented in Figure 3A. We observed that none of the mean values of this A2 region changed significantly by ANOVA ($F = 1.46$, $P = 0.22$), even when we compare pairs of steps from the experimental paradigm (BGM, Stroop Test, IRP, Raven Test, FRP). However, a tendency towards a decrease in this A2 region during the stress tests was observed with a remarkable recovery during the FRP.

The results of the ANOVA for A1 + A2, A3 and the median under A2 + A3 had significant differences among the steps of the experimental paradigm ($F = 3.85$, $P = 0.005$, $F = 7.25$, $P < 0.001$, and $F = 5.5$, $P < 0.001$, respectively). Pairs for the group data analysis are presented in Figure 3B, which shows the results for the region from 1 to 4 cpm (referred to as the extended gastric region). We observed significant changes from the BGM step to the Stroop Test ($P = 0.02$), from the BGM step to the IRP ($P = 0.003$), and from the BGM step to the Raven Test ($P < 0.001$). In addition, we found significant changes from the IRP and Raven Test to the FRP, with $P = 0.01$ and $P = 0.007$, respectively. Similar results to the A1 + A2 region were observed in the A3 region (4-8 cpm), which showed significant changes when the stress psychological tests were administrated from BGM to Stroop Test, IRP, and Raven Test ($P < 0.001$, $P = 0.002$, and $P < 0.001$, respectively) (Figure 3C). There were also significant changes from the IRP to the Raven Test ($P = 0.043$) and from the Raven Test to the FRP ($P = 0.001$) (Figure 3C). These differences were influenced by changes in the adjacent gastric region, A2 (2-4 cpm), and the higher frequency region, A4 (8-12 cpm), but not as much by the decreased ileum activity. As we described in the methodology, one of the parameters that we have proposed in previous studies[16,24] as a useful parameter for EBI gastric studies is the median of the area under the region from 2 to 8 cpm (A2 + A3); namely, the frequency that divides this area into two equal parts. The mean values of these

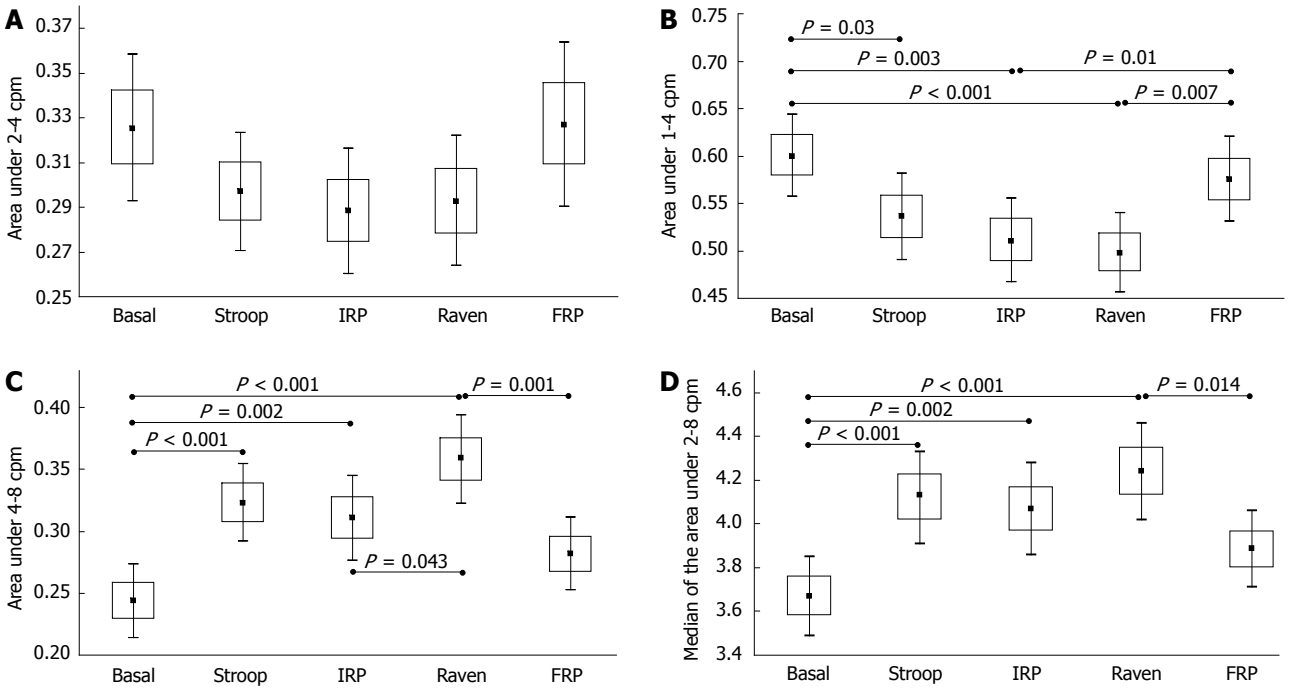


Figure 3 Changes in the relative area under the frequency spectrum of the gastric electrical bio-impedance signal (mean ± SE). A: From 2 to 4 cpm; B: From 1 to 4 cpm; C: From 4 to 8 cpm; D: Median of the area from 2 to 8 cpm. Basal: Basal gastric motility recording; Stroop: Recording during the Stroop Test; IRP: Intermediate resting period; Raven: Recording during the Raven test; FRP: Final resting period.

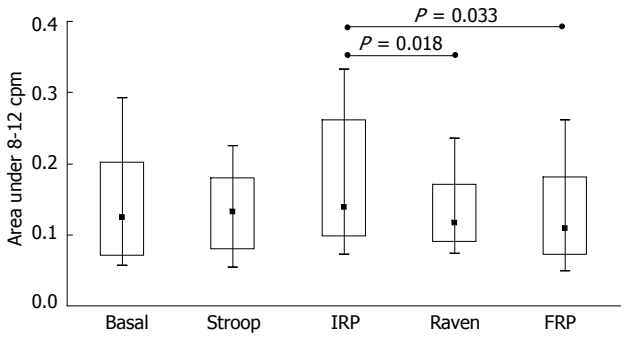


Figure 4 Changes in the relative area under the frequency spectrum of the gastric electrical bio-impedance signal from 8 to 12 cpm (median and interquartile range). Basal: Basal gastric motility recording; Stroop: Recording during the Stroop Test; IRP: Intermediate resting period; Raven: Recording during the Raven Test; FRP: Final resting period.

parameters (median of the A2 + A3 area) are presented in Figure 3D, which also shows the statistical changes when the stress psychological tests were administered, with significant variations from the BGM step to the Stroop Test, IRP and Raven Test ($P < 0.001$, $P = 0.002$, and $P < 0.001$, respectively). We also observed significant changes from the Raven Test to the FRP ($P = 0.014$).

The results of the A4 (8-12 cpm) Friedman ANOVA did not show a significant difference ($\chi^2 = 8.44$, $P = 0.077$). The comparisons among the groups of variables are presented in Figure 4, which gives the results of the Wilcoxon T test for the A4 area. This region, which was influenced by the breathing response, changed significantly during the Raven Test, with significant changes from the IRP step to the Raven Test and from the IRP to the FRP ($P = 0.018$ and $P = 0.033$, respectively).

DISCUSSION

We used the short-term EBI technique to evaluate the physiological changes in gastric activity due to psychological stress caused by the Stroop[20,21] and Raven[22] Tests in healthy women. Several studies have shown that gastric motility alterations are good clinical indicators of diseases[18,25-28]. Using EGG and EBI as noninvasive techniques for assessing gastric motility, we can ensure that the accuracy and reliability of the test and the comfort of the patient are the prevailing conditions under which each patient is assessed[29-32].

As described above, the most widely used parameter for the assessment of gastric motility is the highest peak of activity in the region from 2 to 4 cpm of the frequency spectrum. This parameter could not be trusted in our case because the EBI recorded information from other parts of the GI tract and other systems (breathing)[33-35] strongly interfered with the desired signal. In addition, the short-term EBI recording prevented us from relying on a parameter as being punctual in the low frequency region (2-4 cpm). The area under a specific frequency region is expected to give the relative level of activity in that frequency region without needing the details of the spectrum. An alternative to the EBI gastric motility measurement of two or more areas is the recording of the median of the entire area. In this study, the changes found in gastric motility, as measured by the relative areas, were considered to have statistical significance when P was $\leqslant 0.05$, and in other cases, we included the graph

only to present the tendencies that were physiologically compatible with what we expected as a response to gastric stress[24].

Taking the basal gastric measurement as a reference, we observed that the Stroop Test induced changes in the A1 + A2, A3 and the median of the A2 + A3 parameters (Figure 3B-D), in agreement with the inhibition of gastric activity during psychological stress. We hypothesized that the decrease in the activity in the gastric region produced the increase in the A3 region (Figure 3C). The region from 8 to 12 cpm did not show significant changes during the Stroop Test.

In the intermediate resting period, we observed a slight recovery in the A3 and A4 regions and in the median of the A2 + A3 region; the A1 + A2 region showed a continuing effect of the Stroop Test. None of these changes were statistically significant. During the Raven Test, the A3 region changed significantly by a strengthened effect of the stress caused by the Stroop Test. This effect was also observed in the A4 region. The final resting period change was highly significant in the regions A1 + A2 and A3 and in the median of A2 + A3.

Some general observations should be addressed at this point. All of the parameters consider relative areas under the whole region from 1 to 12 cpm or the median frequency of a relative area; thus, a change in any of these parameters is not only due to the local change, but is also influenced by the changes in the other parameters. The apparent stability of the relative area of A4, which represents approximately 15% of the total area and which includes the breathing frequency, is the result of the breath going beyond the higher frequency limit (12 cpm) and the real decrease in the GI activity (A1 + A2). We believe that the continuing effects of stress after completing the Stroop Test were due in part to the knowledge of the participants that they would receive a second test, which predisposed them towards having a continuous stress response (Figures 3B-D and 4). The immediate recovery of the participants at the end of the Stoop and Raven Tests, as shown by the evaluated parameters reaching the basal values, was likely affected by the knowledge of the participants that the test was complete (Figure 3B-D).

Changes due to stress vary between participants. Typically, the stress starts at the beginning of the psychological test, with a variable reaction time, duration and intensity[24]. Gastric motility changes, with an acceptable physiological explanation, give a clear indication that short-term gastric EBI recording could be useful in routine studies of the response to psychological stress. Further research should address alternative signal analysis approaches, validation, movement discrimination, limitations, variability factors, the normal range of significant parameters, and assessment protocols.

Psychological stressors may have different patterns, accompanied by increases in the sympathetic nervous system activity and total blood vessel peripheral resistance[36]. Therefore, more cardiovascular activity is involved in a specific task that requires active action (as in the Stroop Test) than in those that are considered passive, maintaining a close similarity to the response to a real situation. After using the Stroop Test as a stressor, there was an increase in anxiety, heart rate, respiration, electrodermal activity, muscle tension and the levels of catecholamines, among other psychophysiological variables. The Stroop Test includes complex neuropsychological processes related to the ability to classify information, act selectively, and block non-requested information linked to frontal lobe functions. It is based on the observation that naming colors occurs noticeably slower than reading words. This test assesses some cognitive functions, such as attention, mental flexibility, and the inhibition of automatic responses, related to executive functions associated with the frontal lobes. The other test that has been certified widely in the literature for its usefulness is the Raven Test; however, it is limited by the verbal and cultural influences, level of schooling, economic level, and age. Taken together, the results presented here indicate that short-term gastric EBI has great potential to become a user-friendly methodology to assess gastric motility, particularly with respect to the evaluation of the acute stress response.

In conclusion, the results from this study, which was performed in healthy women, clearly demonstrated that using different parameters, such as the relative areas under the frequency spectrum and the analysis of the median of those areas, in short-term recordings of gastric EBI may be useful for the evaluation of the sympathetic nervous system response to acute psychological stress. The relatively simple signal analysis required for the gastric EBI could make this technique a good candidate for a basic clinical evaluation and even an ambulatory method to assess gastric motility. One of the main advantages of this technique is that it can be implemented using small and inexpensive devices once the frequency and amplitude of the stimulation are known.

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COMMENTS

Background

The relationship between emotions and gastric motility has been documented since the last century. Likewise, psychological stress produces alterations in gastrointestinal tract response in humans. There are several methods for measuring gastric response to stress, but many of these methods are invasive. In this study, authors proposed a novel technique, which was inexpensive and easy to manage, to measure the gastric response to psychological stress by electrical bio-impedance (EBI).

Research frontiers

In healthy humans, anger, fear, painful stimuli, preoperative anxiety and intense exercise are some of the causes of slow gastric emptying. The principal noninvasive techniques to evaluate the gastric motility response during psychological stress are electrogastrography (EGG) and the EBI. In these two techniques, the main challenge is the interpretation of the signal due to the overlapping information from different regions in the gastric system, particularly with respect to lung region movements in the case of EBI; in both cases, different motility

frequencies are involved.

Innovations and breakthroughs

The authors confirm that the gastric response to acute psychological stress can be evaluated by short-term EBI.

Applications

The short-term technical EBI is useful for measuring the gastric response to psychological stress. Besides, it may be useful for frequent monitoring of health status of patients with gastric disorders; being an easy-to-use method of clinical monitoring and diagnosis.

Terminology

EGG is a technique to record the myoelectrical activity of a specific inner surface of the gastrointestinal tract. EGG is a noninvasive technique, in which the electrodes are placed on the skin. EBI records the motility of the stomach using cutaneous electrodes placed in the gastric region. Stroop and Raven Tests are valid and reliable tests to produce psychological stress.

Peer review

This study clearly demonstrated that short-term EBI may be useful for evaluating the gastric activity response to acute psychological stress.

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ORIGINAL ARTICLE

Interplay of neuropilin-1 and semaphorin 3A after partial hepatectomy in rats

Ling Fu, Tsuneo Kitamura, Kazuhisa Iwabuchi, Syozo Ichinose, Mitsuaki Yanagida, Hideoki Ogawa, Sumio Watanabe, Toshihide Maruyama, Masafumi Suyama, Kenji Takamori

Ling Fu, Tsuneo Kitamura, Kazuhisa Iwabuchi, Syozo Ichinose, Mitsuaki Yanagida, Hideoki Ogawa, Kenji Takamori, Institute for Environmental and Gender Specific Medicine, Juntendo University Graduate School of Medicine, Urayasu 279-0021, Japan
Tsuneo Kitamura, Toshihide Maruyama, Masafumi Suyama, Department of Gastroenterology, Juntendo University Urayasu Hospital, Urayasu 279-0021, Japan
Tsuneo Kitamura, Sumio Watanabe, Toshihide Maruyama, Masafumi Suyama, Department of Gastroenterology, Juntendo University School of Medicine, Tokyo 113-8421, Japan
Author contributions: Fu L and Kitamura T designed the research and wrote the paper; Iwabuchi K and Yanagida M revised the article; Fu L and Ichinose S performed the experiments; Ogawa H, Watanabe S, Maruyama T, Suyama M and Takamori K supervised and approved the final publication.
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Correspondence to: Tsuneo Kitamura, MD, PhD, Department of Gastroenterology, Juntendo University Urayasu Hospital, 2-1-1 Tomioka, Urayasu 279-0021, Japan. kitamura@juntendo.ac.jp
Telephone: +81-47-3533111 Fax: +81-47-3815079
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Abstract

AIM: To elucidate the role of neuropilin-1 (Nrp-1) and semaphorin 3A (Sema3A) in sinusoidal remodeling during liver regeneration in rats.

METHODS: Male Wistar/ST rats at 7 wk of age, weighing about 200 g, were used for all animal experiments. *In vivo*, at 24, 48, 72, 96, 144 and 192 h after two-thirds partial hepatectomy (PHx), the remnant livers were removed. Liver tissues were immunohistochemically stained for Nrp-1, Sema3A and SE-1, a liver sinusoidal endothelial cell (SEC) marker. Total RNA of the liver tissue was extracted and reversely transcribed into cDNA. The mRNA expression of Sema3A was analyzed by quantitative real-time polymerase chain reaction and normalized to that of ribosomal protein S18. *In vitro*, SECs were isolated from rat liver and cultured in endothelial growth medium containing 20 ng/mL vascular endothelial cell growth factor. Migration of SECs in primary culture was assessed by cell transwell assay with or without recombinant Sema3A. Apoptotic cells were determined by a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling method.

RESULTS: *In vitro*, immunohistochemistry study revealed that Sema3A and Nrp-1 were constitutively expressed in hepatocytes and SECs, respectively, in normal rat liver tissues. Nrp-1 expression in SECs was quantified by the percentage of immunostained area with anti-Nrp-1 antibody in relation to the area stained with SE-1. Between 24 h and 96 h following resection of liver, Nrp-1 expression in SECs was transiently increased. Compared with the baseline (5.2% ± 0.1%), Nrp-1 expression in SECs significantly increased at 24 h (17.3% ± 0.7%, $P < 0.05$), 48 h (39.1% ± 0.6%, $P < 0.01$), 72 h (46.9% ± 4.5%, $P < 0.01$) and 96 h (29.9% ± 3.8%, $P < 0.01$) after PHx, then returned to the basal level at termination of liver regeneration. Interestingly, the expression of Sema3A was inversely associated with that of Nrp-1 in liver after PHx. Sema3A mRNA expression was significantly reduced by about 75% over the period 24-144 h after PHx ($P < 0.05$), and returned to basal levels at 192 h after PHx. *In vitro*, SECs isolated from rats after PHx (PHx-SECs) were observed to migrate to the lower chamber of the cell transwell system after incubation for 24 h, but not cells from normal rats (CONT-SECs), indicating that mobility of PHx-SECs increases as compared with that of CONT-SECs. Moreover, recombinant Sema3A significantly attenuated migration in PHx-SECs in primary culture (vehicle-treated

100% ± 7.9% *vs* Sema3A-treated 42.6% ± 5.4%, $P < 0.01$), but not in CONT-SECs. Compared with CONT-SECs, the apoptotic rate of PHx-SECs decreased by 78.3% ($P < 0.05$). There was no difference in apoptosis between CONT-SECs that were treated with vehicle and Sema3A. However, in PHx-SECs, apoptosis was induced by the presence of 5 nmol Sema3A for 24 h (vehicle-treated 21.7% ± 7.6% *vs* Sema3A-treated 104.3% ± 8.9%, $P < 0.05$). In addition, immunohistochemistry confirmed the increased expression of Nrp-1 in PHx-SECs, while it was noted to a lesser extent in CONT-SECs.

CONCLUSION: The interplay of Nrp-1 and Sema3A shown in our results may lead to a better understanding of interaction between sinusoidal remodeling and SECs during liver regeneration.

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Key words: Neuropilin-1; Semaphorin 3A; Sinusoidal remodeling; Liver regeneration; Two-thirds partial hepatectomy; Angiogenesis; Liver sinusoidal endothelial cells

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INTRODUCTION

In liver, adult hepatocytes are long-lived and normally do not undergo cell division, but they maintain the ability to proliferate in response to toxic injury or partial resection of liver[1,2]. The process of liver regeneration (LR) is complex and involves a finely orchestrated balance of cell proliferation to reconstruct the normal liver architecture. Although hepatocyte proliferation is a major feature of the regenerating liver, it involves the interactions of other hepatic cell types (non-parenchymal cells), including Kupffer cells, stellate cells, sinusoidal endothelial cells (SECs) and biliary epithelial cells[3-5].

In the rat liver after two-thirds hepatectomy, the rate of DNA synthesis in hepatocytes begins to increase after about 12 h and peaks around 24 h. However, the induction of DNA synthesis occurs later in the non-parenchymal cells (at approximately 48 h for Kupffer and biliary epithelial cells, and at approximately 96 h for SECs), and subsequently, LR terminates at approximately 192 h[6,7]. In the late stage of this process, angiogenesis and/or vasculogenesis is essential to supply blood flow to newly replicating avascular islands of hepatocytes, in which SECs migrate and proliferate, leading to the formation of new functional sinusoids[8,9].

Indeed, many of the growth factors have been reported to be upregulated in a regenerating liver. Vascular endothelial growth factor (VEGF) is a major proangiogenic factor and is thought to improve sinusoid reconstruction during the LR process[10,11]. Hepatocyte growth factor (HGF) levels are dramatically increased after partial hepatectomy, but HGF is also a potent proangiogenic factor *in vivo* and stimulates endothelial cell protease production, motility, proliferation and differentiation *in vitro*.

Recently, the importance of neuropilins (Nrps) as proangiogenic factors has been highlighted[12]. Nrps are transmembrane glycoproteins with large extracellular domains that interact with both class 3 semaphorins and VEGF, and they are involved in the regulation of many physiological pathways including angiogenesis[13,14]. The semaphorins were initially described as axon guidance factors that affect the development of the central nervous system[15]. However, semaphorin receptors were subsequently found to be expressed by multiple types of cells including endothelial cells and cancer cells. These observations were followed by studies that indicate that the semaphorins can modulate the behavior of cancer cells and endothelial cells[16,17].

In this study, we report the inverse expression of Nrp-1 and semaphorin 3A (Sema3A) in liver tissues during liver regeneration in rats. We further show that Sema3A significantly attenuated migration and induced apoptosis in SECs isolated from rats after partial hepatectomy, but not in cells from normal rats.

MATERIALS AND METHODS

Animals and treatment

Male Wistar/ST rats, 7 wk of age, weighing about 200 g (Sankyo lab, Tokyo, Japan), were used for all animal experiments. Two-thirds partial hepatectomy (PHx) was performed under sevoflurane inhalant anesthesia according to the method of Higgins *et al*[18]. The left lateral and median lobes of the liver were excised. At 24, 48, 72, 96, 144 and 192 h after PHx, the rats were anesthetized and the remnant livers were removed. Control livers were obtained from sevoflurane-anesthetized non-manipulated animals.

Liver tissues were obtained from the right lateral lobe, embedded in Tissue Tek OCT Compound (Electron Microscopy Sciences, Hatfield, PA), frozen and stored at -80 ℃ until use. All procedures involving animals were approved by the Animal Care and Use Committee of Juntendo University, which conforms to the National Institutes of Health Guideline.

Immunofluorescent staining

Double staining of Nrp-1 and SE-1 was performed as follows: tissue fixed with 4% paraformaldehyde for 10 min at

room temperature, incubated with goat polyclonal anti-Nrp-1 antibody (R and D Systems, Minneapolis, MN) (diluted 1:50) and mouse monoclonal SE-1 (Immuno-Biological Laboratories, Gunma, Japan) (diluted 1:20) at 4 ℃ overnight prior to incubation with Alexa-488 rabbit anti-goat (diluted 1:300) and Alexa-594 donkey anti-mouse antibody (diluted 1:300) (Invitrogen, Carlsbad, CA) for 1 h, then mounted in Vectashield mounting medium with 4,6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories, Burlingame, CA). The immunostained samples were observed and analyzed with a BIOREVO BZ-9000 microscope system (KEYENCE, Osaka, Japan).

For Sema3A staining, frozen sections of liver tissues were fixed with -20 ℃ acetone for 5 min, incubated with rabbit polyclonal anti-Sema3A antibody (diluted 1:200) (Abcam, Cambridge, United Kingdom) at 4 ℃ overnight prior to incubation with Alexa-488 goat anti-rabbit antibody (diluted 1:300) for 1 h.

Quantitative real-time polymerase chain reaction

Total RNA of the liver tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and reversely transcribed into cDNA using the RT reagent kit (TaKaRa Bio Inc., Shiga, Japan). Quantitative real-time polymerase chain reaction (PCR) was performed using ABI PRISM 7900HT Sequence Detector System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The following primers were used: Sema3A (accession number: NM_017310.1) CTG CTC CGA CTT GCA GCA TC (sense) and CGC CTC TGA AAT TGC CAA TAT ACC (antisense); ribosomal protein S18 (RPS18) (accession number: NM_213557.1) AAG TTT CAG CAC ATC CTG CGA GTA (sense) and TTG GTG AGG TCA ATG TCT GCT TTC (antisense). The Sema3A mRNA expression was normalized to that of RPS18.

Preparation of SECs in primary culture

SECs were isolated from rat liver based on a modification of the method of Braet *et al*[19], as we previously reported[20]. Briefly, liver was perfused with 0.05% collagenase A and 0.001% DNase. SECs were then purified by isopycnic sedimentation in a two-step Percoll gradient (25% and 50%). SECs were cultured in EBM-2 medium containing 20 ng/mL VEGF. These cells displayed a number of fenestrae in sieve plates, the characteristic of SECs *in vivo*. In separate experiments, SECs were isolated from rats 4 d after PHx (PHx-SECs).

Cell transwell migration assay

SEC transwell migration assay was performed using 24-well culture inserts (BD Biosciences, San Jose, CA). Polyethylene terephthalate membranes with a pore size of 8 μm were coated with fibronectin. SECs were loaded to the upper chamber. Recombinant Sema3A (R and D Systems, Minneapolis, MN) or vehicle was added to the lower chamber. After incubation for 24 h, non-migrated cells on the upper surface of the filter were removed with a cotton swab and the cells which traversed and spread on the lower surface of the membrane were fixed with 2.5% glutaraldehyde and stained with hematoxylin. The average number of the cells per field was counted ×10 objective magnification from five microscopic fields. Migration index represents the number of migratory cells/number of migratory cells in vehicle-treated control.

Apoptosis assay

After incubation with 5 nmol recombinant Sema3A for 24 h, SECs were fixed with 4% paraformaldehyde for 10 min at room temperature. Apoptotic cells were determined by a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method with a fluorescent apoptosis detection system kit (Promega, Madison, WI).

Statistical analysis

Results are expressed as the mean ± SE. Statistical analysis were performed using GraphPad Prism v5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was determined by one-way analysis of variance.

RESULTS

Nrp-1 expression in SECs increases in regenerating liver after PHx

To investigate Nrp-1 expression in SECs during LR, liver tissues after PHx were analyzed by immunofluorescent staining with anti-Nrp-1 antibody and SE-1, a monoclonal antibody that specifically reacts with rat liver SECs[21,22]. As shown in Figure 1A, SECs stained with SE-1 were positive for Nrp-1 in control liver, and Nrp-1 expression in SECs was enhanced in regenerating liver at 72 h after PHx.

Nrp-1 expression in SECs was quantified by the percentage of immunostained area with anti-Nrp-1 antibody in relation to the area stained with SE-1. Nrp-1 expression in SECs significantly increased at 24 h ($P < 0.05$), peaked at 72 h ($P < 0.01$), and recovered to the basal level at 144 h after PHx (Figure 1B).

Sema3A expression decreases in regenerating liver after PHx

To test the expression of Sema3A during LR, liver tissues were examined by immunofluorescence with anti-Sema3A antibody in control rats and rats at 72 h after PHx. Sema3A was strongly stained in hepatocytes, which display round and big nuclei, in control liver. However, Sema3A expression was attenuated at 72 h after PHx (Figure 2A). Furthermore, real-time PCR analysis confirmed the decline of Sema3A expression during the process of LR. As shown in Figure 2B, Sema3A mRNA expression was significantly reduced by about 75% over the period 24-144 h after PHx ($P < 0.05$), and returned

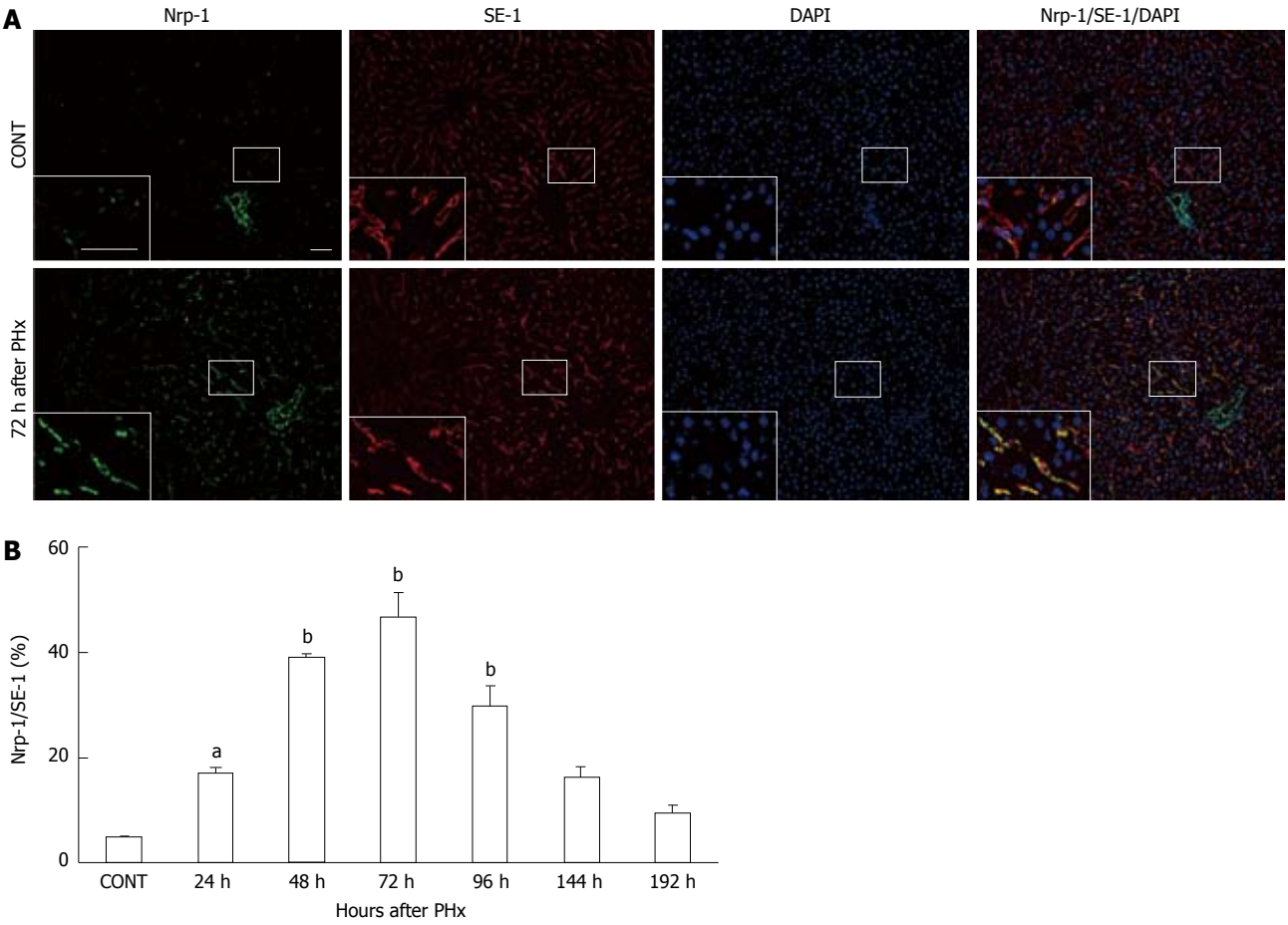


Figure 1 Neuropilin-1 expression in sinusoidal endothelial cells in regenerating liver. A: Immunofluorescent examination of neuropilin-1 (Nrp-1) in control liver and regenerating liver 72 h after partial hepatectomy. Green: Nrp-1; Red: Sinusoidal endothelial cells stained with SE-1; Blue: Nuclei stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Scale bar, 50 μm; B: Graphic representation of the percentage of immunostained area with anti-Nrp-1 antibody in relation to the area stained with SE-1 (%). Results are expressed as the mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control liver ($n = 3$). PHx: Partial hepatectomy.

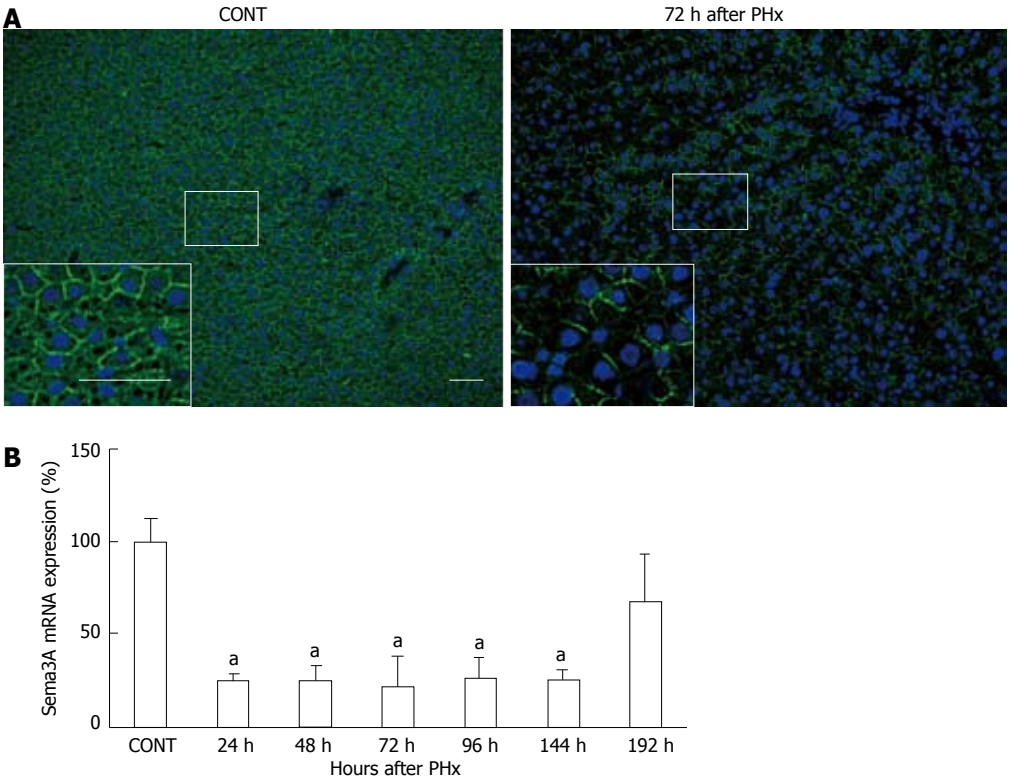


Figure 2 Semaphorin 3A expression in liver tissues after partial hepatectomy. A: Immunofluorescent examination of semaphorin 3A (Sema3A) in control liver and regenerating liver 72 h after partial hepatectomy (PHx). Green: Sema3A; Blue: Nuclei stained with 4,6-diamidino-2-phenylindole dihydrochloride. Scale bar, 50 μm; B: Sema3A mRNA expression in liver tissues after PHx. Sema3A mRNA expression was quantified by real-time polymerase chain reaction and normalized to ribosomal protein S18 mRNA level. Results are expressed as the mean ± SE. $^{a}P < 0.05$ *vs* control liver ($n = 3$).

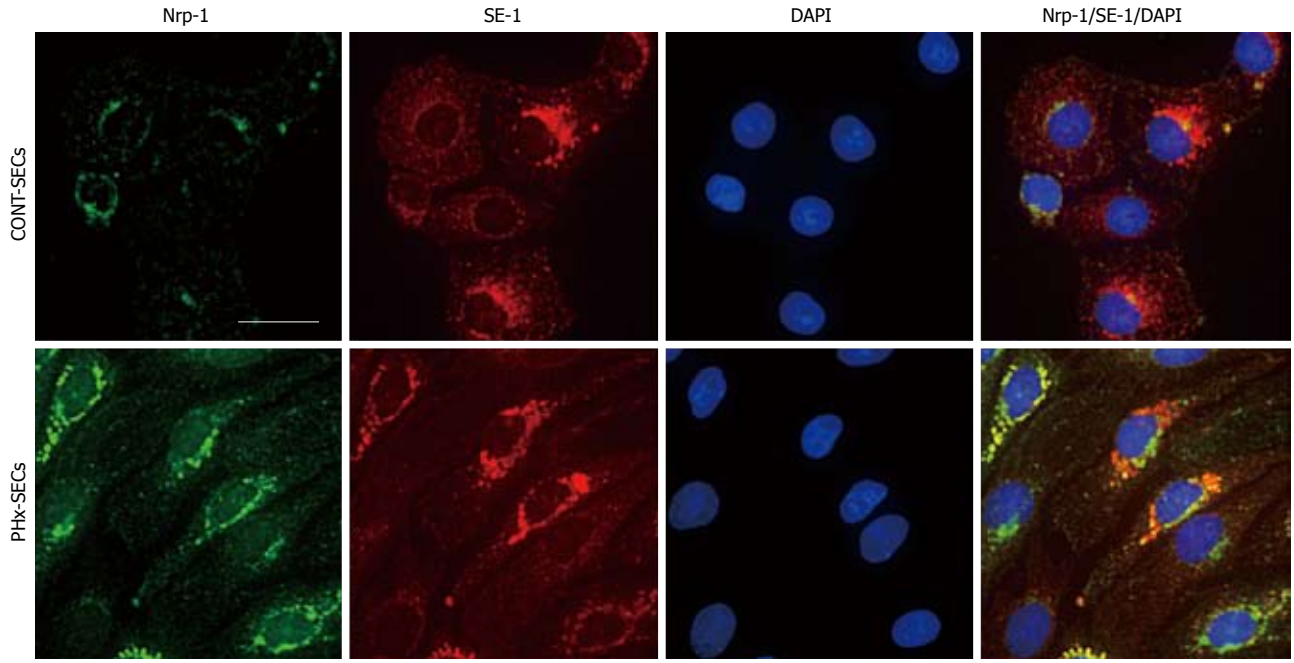


Figure 3 Immunofluorescent examination of neuropilin-1 and SE-1 in sinusoidal endothelial cells in primary culture. A: Neuropilin-1 (Nrp-1) expression in sinusoidal endothelial cells (SECs) isolated from normal rats (CONT-SECs); B: Nrp-1 expression in SECs isolated from rats after partial hepatectomy (PHx-SECs). Green: Nrp-1; Red: SE-1; Blue: Nuclei stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Scale bar, 20 μm.

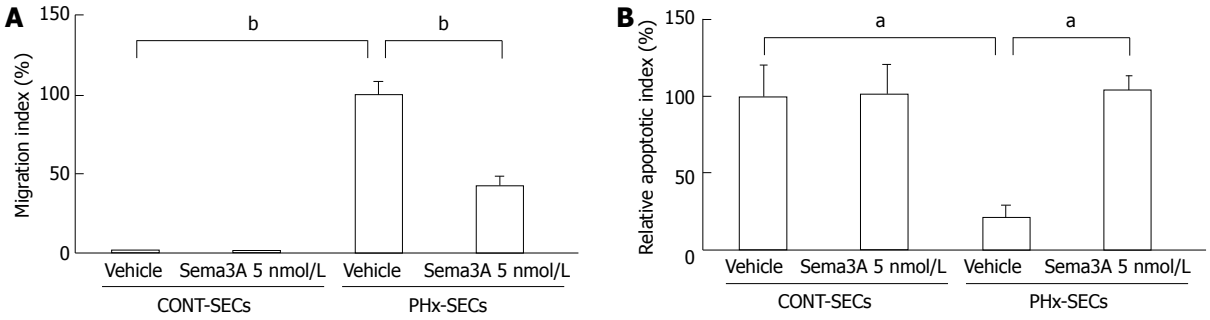


Figure 4 Role of semaphorin 3A in sinusoidal endothelial cell migration and apoptosis. A: Effect of semaphorin 3A (Sema3A) on migration of sinusoidal endothelial cells (SECs) isolated from normal rats (CONT-SECs) and rats after partial hepatectomy (PHx-SECs). SEC migration was assessed by cell transwell assay in the presence or absence of 5 nmol Sema3A for 24 h. Migration index represents the number of migratory cells/number of migratory cells in vehicle-treated PHx-SECs; B: Effect of Sema3A on apoptosis of CONT-SECs and PHx-SECs. Following incubation of SECs with 5 nmol Sema3A or vehicle for 24 h, apoptotic cells were determined by a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling method. Results are expressed as the mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* vehicle-treated PHx-SECs ($n = 4$).

to baseline at 192 h after PHx.

Nrp-1 expression increases in PHx-SECs as compared with CONT-SECs in primary culture

To confirm that Nrp-1 is expressed in SECs, immunofluorescent analysis was performed in SECs isolated from rat liver. Both CONT-SECs and PHx-SECs at 24 h in culture were stained positive for Nrp-1 and SE-1, and Nrp-1 expression was increased in PHx-SECs as compared with CONT-SECs (Figure 3A and B). The up-regulated Nrp-1 expression in PHx-SECs was consistent with *in vivo* data shown in Figure 1. Although fluorescence is observed in the perinuclear region as well as the plasma membrane, this may suggest that Nrp-1 presents intracellular trafficking between plasma membrane and intracellular compartments, such as endoplasmic reticulum, Golgi, endosomes and lysosomes.

Sema3A attenuates migration and elicits apoptosis of PHx-SECs

To investigate the effect of Sema3A on SEC mobility, migration of cells was quantified by cell transwell assay in SECs isolated from rats after PHx (PHx-SECs) and normal rats (CONT-SECs). As a result, PHx-SECs were observed to migrate to the lower chamber after incubation for 24 h, but not CONT-SECs, indicating that mobility of PHx-SECs increases as compared with that of CONT-SECs. Moreover, 5 nmol Sema3A significantly attenuated migration of PHx-SECs by 57% ($P < 0.01$, Figure 4A), while it did not affect proliferation of PHx-SECs (data

not shown).

Based on previous reports showing that Sema3A induces apoptosis[23-25], we explored the effect of Sema3A on apoptotic rate in PHx-SECs and CONT-SECs with TUNEL assay. Compared with CONT-SECs, apoptotic rate in PHx-SECs decreased by 78% ($P < 0.05$, Figure 4B). There was no difference in apoptosis between CONT-SECs that were treated with vehicle and Sema3A. However, in PHx-SECs, apoptosis was induced by the presence of 5 nmol Sema3A for 24 h ($P < 0.05$, Figure 4B).

DISCUSSION

The adult hepatocyte is normally a quiescent, highly differentiated cell, but almost immediately it can begin the process of replication in response to conditions that induce cell loss by physical, infectious, or toxic injury. The events involved in the regenerative response are precise, carefully orchestrated, and highly regulated. Indeed, the hepatocyte is a key player in the process of LR, but other cell types, including non-parenchymal cells, also play important roles in reformation of the normal liver architecture.

Among non-parenchymal cells, SECs line the intrahepatic circulatory vessels, named sinusoids, and provide a large surface area for nutrient absorption. Thus, in regenerating liver, SECs play a central role in reconstruction of sinusoids through which blood flow is supplied into the hepatocytes[8,9,26]. Previous reports using electron microscopy have shown that SECs proliferate and migrate into initially formed avascular clusters of proliferating hepatocytes, leading to the formation of new functional sinusoids[9].

In the process of sinusoidal remodeling, various proangiogenic factors are produced in response to upregulation of hypoxia-inducible factor, which is generated within the avascular clusters of hepatocytes. Among these, vascular endothelial growth factor (VEGF) has been shown to be a key regulator of hepatic neovascularization, and is involved in three tyrosine kinase receptors (VEGFR1, R2, and R3), and two non-tyrosine kinase receptors (Nrp-1 and Nrp-2) in inducing signal transduction[27].

Neuropilins were first identified as receptors for class 3 semaphorins, a family of soluble molecules with neuronal guidance functions[28,29]. During development, Nrp-1 is preferentially expressed in arteries, and Nrp-2 is expressed in veins and lymphatics[30]. There is a degree of specificity in semaphorin/neuropilin binding and activity, with semaphorin 3A (Sema3A) binding to Nrp-1, Sema3F binding to Nrp-2 and Sema3B binding to both receptors on endothelial cells[13]. Recently it has been suggested that these semaphorins may interact with the vasculature in an inhibitory manner. Indeed, *in vitro* studies suggest that class 3 semaphorins compete with VEGF for Nrp-1/Nrp-2 binding, thereby inhibiting mitogenic effects of VEGF in endothelial cells, and leading to apoptosis and inhibition of migration in breast cancer cell lines[31,32].

In this study, to investigate interplay of Nrp-1 and Sema3A in sinusoidal remodeling during LR, we examined the expression of Nrp-1 in liver tissues after PHx in rats, and found that Nrp-1 is constitutively expressed in SECs in normal liver, but its expression is increased following resection of liver, returning to the basal level at termination of liver regeneration. This is consistent with well-documented findings that, following hepatocyte proliferation, DNA synthesis of SECs begins in the late stage of LR[2,7]. Increased expression of Nrp-1 was further confirmed by *in vitro* study with SECs isolated from rats after PHx, while it was noted to a lesser extent in cells from normal liver.

Interestingly, the inverse expression of Sema3A and Nrp-1 was observed in liver during the process of LR. Immunohistochemistry also indicated the expression of Sema3A in hepatocytes, suggesting the possibility that Sema3A may interact with Nrp-1 that is expressed in SECs. Indeed, our *in vitro* results show that Sema3A significantly attenuated migration and induced apoptosis in SECs isolated from rats after PHx, but not in cells from normal rats.

Recently, many studies have illustrated the crucial role of Nrp-1 in cell proliferation in development and cancer. The absence of a functional Nrp-1 receptor results in embryonic lethality as a result of impaired heart and blood vessel development, indicating that this receptor plays a central regulatory role during developmental angiogenesis[33]. Furthermore, in endothelial-specific Nrp-1 knockouts, certain arterial markers are missing from arterioles and arteries[34,35]. Alternatively, a selective Sema3A inhibitor has been shown to enhance regenerative responses and functional recovery of the injured spinal cord[36].

Although further studies are required to reveal the underlying mechanism, our results showing inverse expression of Nrp-1 and Sema3A in liver after PHx may provide a better understanding of sinusoidal remodeling in LR.

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COMMENTS

Background

The process of liver regeneration (LR) is complex and involves a finely orchestrated balance of cell proliferation to reconstruct the normal liver architecture. Although hepatocyte proliferation is a major feature of the regenerating liver, it involves the interactions of other hepatic cell types (non-parenchymal cells), including Kupffer cells, stellate cells, sinusoidal endothelial cells and biliary epithelial cells.

Research frontiers

Many of the growth factors have been reported to be upregulated in a regenerating liver. Vascular endothelial growth factor (VEGF) is a major proangiogenic

factor and is thought to improve sinusoid reconstruction during the LR process.

Innovations and breakthroughs

Recently, the importance of neuropilins (Nrps) as proangiogenic factors has been highlighted. Nrps are transmembrane glycoproteins with large extracellular domains that interact with both class 3 semaphorins and VEGF, and are involved in the regulation of many physiological pathways including angiogenesis. Semaphorin receptors were subsequently found to be expressed by multiple types of cells including endothelial cells.

Applications

The findings of this study may offer new insights into the role of Nrp-1 and semaphorin 3A (Sema3A) as a target for novel therapeutic strategies in impaired liver regeneration.

Terminology

Nrp-1 was originally characterized as a Sema3A receptor regulating axon guidance, and it was later demonstrated that Nrp-1 is also important for vascular morphogenesis. Nrp-1 acts as receptor for VEGF, whereby it serves to promote VEGF-induced angiogenesis. Because Sema3A inhibits angiogenesis through Nrp-1, it has been suggested that it acts by merely competing with VEGF and thus inhibiting its proangiogenic effects.

Peer review

The authors present interesting results on the interaction of neuropilin and semaphorin in control of angiogenesis during liver regeneration. Overall, the results presented support the conclusions.

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ORIGINAL ARTICLE

Tumor necrosis factor alpha increases intestinal permeability in mice with fulminant hepatic failure

Guo-Zhen Li, Zhao-Han Wang, Wei Cui, Jin-Long Fu, Yu-Rong Wang, Pei Liu

Guo-Zhen Li, Zhao-Han Wang, Wei Cui, Jin-Long Fu, Yu-Rong Wang, Pei Liu, Department of Infectious Diseases, the First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China

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Correspondence to: Dr. Pei Liu, Department of Infectious Diseases, the First Affiliated Hospital, China Medical University, No. 155 Nanjing Street, Shenyang 110001, Liaoning Province, China. syliupei2003@yahoo.com.cn
Telephone: +86-24-83283091 Fax: +86-24-83282805

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Abstract

AIM: To determine the effect of tumor necrosis factor alpha (TNF-α) on intestinal permeability (IP) in mice with fulminant hepatic failure (FHF), and the expression of tight junction proteins.

METHODS: We selected D-lactate as an index of IP, induced FHF using D-galactosamine/lipopolysaccharide and D-galactosamine/TNF-α, assessed the results using an enzymatic-spectrophotometric method, transmission electron microscopy, immunohistochemistry, Western blotting and real-time quantitative polymerase chain reaction. The effect of the administration of anti-TNF-α immunoglobulin G (IgG) antibody, before the administration of D-galactosamine/lipopolysaccharide, on TNF-α was also assessed.

RESULTS: IP was significantly increased in the mouse model of FHF 6 h after injection (13.57 ± 1.70 mg/L, 13.02 ± 1.97 mg/L *vs* 3.76 ± 0.67 mg/L, $P = 0.001$). Electron microscopic analysis revealed tight junction (TJ) disruptions, epithelial cell swelling, and atrophy of intestinal villi. Expression of occludin and claudin-1 mRNA was significantly decreased in both FHF models (occludin: 0.57 ± 0.159 fold *vs* baseline, $P = 0.000$; claudin-1: 0.3067 ± 0.1291 fold *vs* baseline, $P = 0.003$), as were the distribution density of proteins in the intestinal mucosa and the levels of occludin and claudin-1 protein (occludin: 0.61 ± 0.0473 fold *vs* baseline, $P = 0.000$; claudin-1: 0.6633 ± 0.0328 fold *vs* baseline, $P = 0.000$). Prophylactic treatment with anti-TNF-α IgG antibody prevented changes in IP (4.50 ± 0.97 mg/L *vs* 3.76 ± 0.67 mg/L, $P = 0.791$), intestinal tissue ultrastructure, and the mRNA levels of occludin and claudin-1 expression (occludin: 0.8865 ± 0.0274 fold *vs* baseline, $P = 0.505$; claudin-1: 0.85 ± 0.1437 fold *vs* baseline, $P = 0.1$), and in the protein levels (occludin: 0.9467 ± 0.0285 fold *vs* baseline, $P > 0.05$; claudin-1: 0.9533 ± 0.0186 fold *vs* baseline, $P = 0.148$).

CONCLUSION: Increased in IP stemmed from the downregulation of the TJ proteins occludin and claudin-1, and destruction of the TJ in the colon, which were induced by TNF-α in FHF mice.

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Key words: Tumor necrosis factor alpha; Fulminant hepatic failure; Intestinal permeability; Occludin; Claudin-1

Peer reviewers: Tamara Vorobjova, Senior Researcher, Department of Immunology, University of Tartu, Ravila 19, 50411 Tartu, Estonia; Zhao-Xiang Bian, Professor, School of Chinese Medicine, Hong Kong Baptist University, 1/F Jockey Club School of Chinese Medicine Building, 7 Baptist University Road, Kowloon Tong, Hong Kong, China

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INTRODUCTION

Fulminant hepatic failure (FHF) is a devastating disease with high mortality. The intestinal mucosal barrier plays an important role in the body's protection against luminal pathogens and antigenic molecules. Increased intestinal permeability (IP) and disruption of the intestinal mucosal barrier have been observed in many hepatic diseases[1-4] that cause intestinal endotoxemia (IETM), also called gut-derived endotoxin. High levels and incidence of IETM have been reported in many hepatic diseases[5,6]. The IETM results in excessive inflammatory responses, with serious hepatic necrosis, further severe hepatitis, and even accelerated liver failure.

There are two potential pathways for passive permeation across the intestinal epithelia: the transcellular pathway (through the cells) and the paracellular pathway (between cells). The tight junction (TJ) is a semipermeable barrier that allows certain solutes (depending upon their size and charge) to pass through the paracellular pathway between the cells from one fluid component to the other, and even regulates the permeability of the paracellular spaces. It is thought that TJ may be functionally altered in model disease states, even when the intestinal epithelium remains confluent[7]. Structurally, TJs are composed of cytoplasmic proteins, including the zonula occludens (ZO) proteins, ZO-1, ZO-2, and ZO-3[8,9], as well as two distinct transmembrane proteins, occludin and claudins[10,11], especially claudin-1, the most widely distributed protein in the intestinal epithelium. Occludin and claudins are the major transmembrane proteins that interact with intracellular plaque proteins such as ZO-1, ZO-2, and ZO-3, which in turn interact with the actin cytoskeleton to anchor occludin and other transmembrane proteins at the apical end of the lateral membrane[1]. It has been shown that TJ proteins and IP could be modulated by many factors, including shock[12], hyperthermia[13], alcoholic or nonalcoholic liver disease[2,3], and referred to the cytokines, tumor necrosis factor alpha (TNF-α), and interleukin (IL)-1, and nitric oxide (NO)[14-17]. There are few studies on IP and its mechanism of modulation in FHF.

TNF-α is a key pro-inflammatory cytokine with a broad-spectrum of effects, and is primarily secreted by monocytes and macrophages. It can induce a hepatocyte necrosis *in vivo* or *in vitro*, and is elevated in FHF[18,19]. TNF-α can increase IP *in vitro*[14,17]; thus its role in the possible alteration of IP in FHF should be considered. D-lactate is produced by indigenous bacteria in the gastrointestinal tract[20]. Mammals do not have the enzyme systems to rapidly metabolize D-lactate. Evaluation of D-lactate levels indicates readily the change of IP[21]. We have tested TNF-α concentrations and intestinal ZO-1 expression in the FHF model in mice[22]. In this study, we utilized the same model and controls to investigate the changes in the permeability of the intestinal mucosa and attempted to elucidate the underlying molecular mechanisms by examining TJ ultrastructure and by measuring the expression of the TJ proteins, occludin, and claudin-1.

MATERIALS AND METHODS

Animals and treatment

Male, six-to eight-week-old BALB/c mice (China Medical University) were obtained from the China Medical University (Shenyang, China). They were housed and cared for in rooms maintained at a constant temperature and humidity. Food and water were allowed ad libitum. The mice were fasted overnight for the experiment. All animal experimental procedures were approved by the Ethics Committee of China Medical University before the commencement of the study. All mice were randomly divided into eight groups (n = 7 per group). One group of mice was given intraperitoneal injections of D-galactosamine (GalN; 800 mg/kg body weight; Sigma, Saint Louis, United States) and lipopolysaccharide (LPS; 10 μg/kg body weight; Sigma) to induce acute liver failure (ALF). A second ALF-induction group was also given intraperitoneal injections of GalN (800 mg/kg body weight) and TNF-α (10 μg/kg body weight; Sigma). First group was given anti-TNF-α immunoglobulin G (IgG) (100 μg per mouse; US Biological, United States) antibody treatment prior to ALF induction. The anti-TNF-α IgG antibody was injected *via* the vena caudalis 30 min before GalN/LPS administration. There were four control groups, which were injected intraperitoneally with GalN, LPS, TNF-α, or normal saline (NS). In summary, the eight groups were: (1) GalN/LPS; (2) GalN/TNF-α; (3) GalN control; (4) LPS control; (5) TNF-α control; (6) NS control; and (7) anti-TNF-α antibody and GalN/LPS (named anti-TNF-α IgG antibody pretreated group). Mice in first group were euthanized 2, 6, 9, 12 and 24 h after treatment. Other aforementioned groups were euthanized 6 h after administration of GalN/LPS.

D-lactate determination

The plasma from systemic blood samples was obtained and subjected to a deproteination and neutralization process by acid/base precipitation using perchloric acid and potassium hydroxide. The protein-free plasma was then assayed for D-lactate concentration by enzymatic-spectrophotometric method[23].

Detection and observation of colonic mucosal ultrastructure

Ultrathin (70 nm) colon sections were examined using a transmission electron microscope (Hitachi H-600, Japan).

Immunohistochemistry of occludin, claudin-1

Frozen colon tissue sections (5 μm thick) were fixed on glass slides by incubating them in acetone for 10 min at 4 ℃. The slides were incubated with 3% H_2O_2 for 20 min at room temperature and indirectly immunolabeled using an ABC kit (Takara, Japan) according to the manufacturer's instructions. Slides were then blocked in goat serum for 30 min at 37 ℃ and incubated with a rabbit anti-mouse polyclonal occludin, claudin-1 antibody (dilution, 1:50; Santa Cruz Biotechnology, United States) at

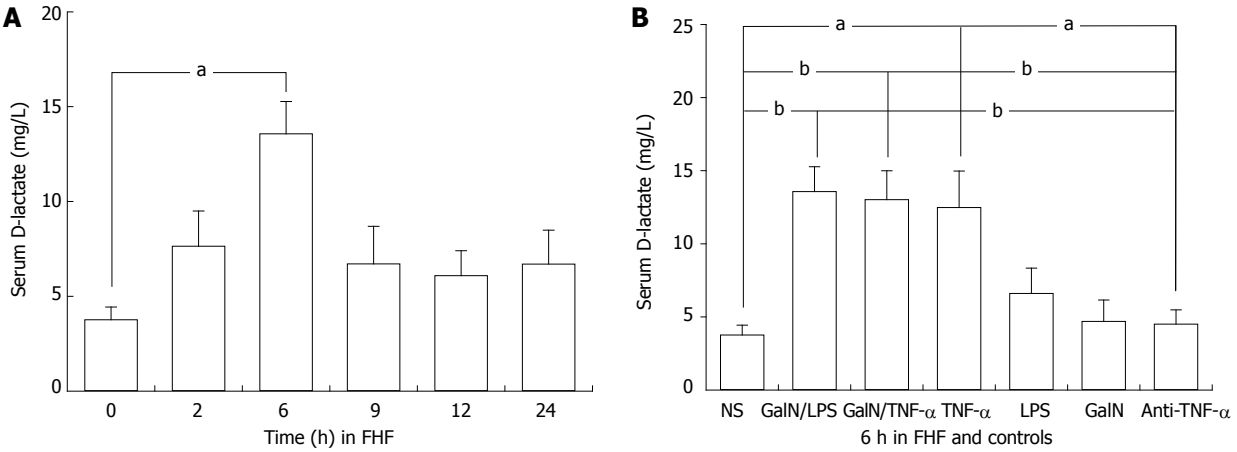


Figure 1 Systemic blood D(-)-lactate levels in the mouse model of fulminant hepatic failure and controls. Systemic blood D (-)-lactate levels 2, 6, 9, 12 and 24 h after injection in D-galactosamine (GalN)/lipopolysaccharide (LPS)-treated mice, and 6 h after injection in the saline, GalN, LPS, tumor necrosis factor alpha (TNF-α), anti-TNF-α immunoglobulin G antibody-pretreated groups. *n* = 5 rats for each time point. NS: Normal saline; FHF: Fulminant hepatic failure. The level of significance was set at $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* baseline (NS group). Data are reported as the mean ± SD.

4 ℃ overnight. For the negative controls, the primary antibody was replaced with phosphate buffered saline (PBS). This incubation was followed by incubation with biotinylated goat anti-rabbit IgG (Histostain-Plus kit, ZYMED) diluted 1:300 in PBS for 2 h at room temperature. Sections were rinsed in PBS and then in distilled water. The slides were stained with 3,3'-diaminobenzidine and counterstained with hematoxylin.

Western blotting analysis of occludin, claudin-1

Intestinal tissue samples were homogenized in lysis buffer [20 mmol Tris-HCl (pH 7.5), 1% Triton X 100, 0.2 mol NaCl, 2 mmol ethylene diamine tetraacetic acid, 2 mmol ethylene glycol tetraacetic acid, 1 mol dithiothreitol and 2 mol aprotinin]. Proteins (50 μg) were electrophoresed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (8%) and transferred to a nitrocellulose membrane. Membranes were blocked with non-fat dried milk in tris buffered saline containing 0.05% Tween-20 (TTBS) for 1 h at room temperature and incubated with a rabbit anti-mouse polyclonal occludin, claudin-1 antibody (diluted 1:400; Santa Cruz Bio-technology) at 4 ℃ overnight. After three washes in TTBS, the membranes were reacted with a 1:2000 dilution of alkaline phosphatase-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology) for 2 h at room temperature. The immunoreaction was visualized using α-dianisidine and β-naphthyl acid phosphate (Sigma, United States).

RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was isolated from intestinal tissues using TRIzol Reagent (Invitrogen, United States). RNA was purified using DNase I and depurified using PI-PCI-EHCO. SYBR-green-based real-time polymerase chain reaction (PCR) [TaKaRa SYBR reverse transcription-PCR kit, Japan] was used to measure relative gene expression in each sample. PCR was performed using *Taq* DNA polymerase (Qiagen, Valencia, United States) and oligonucleotide primers for mouse were occludin (forward 5'-GCTTATCTTGGGAGCCTGGACA-3', reverse 5'-GTCATTGCTTGGTGCATAATGATTG-3'), claudin-1 (forward 5'-AGACCTGGATTTGCATCTTGGTG-3', reverse 5'-TGCAACATAGGCAGGACAAGAGTTA-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH: forward 5'-TGTGTCCGTCGTGGATCTGA-3', reverse 5'-TTGCTGTTGAAGTCGCAGGAG -3'). PCR conditions were as follows: one cycle at 95 ℃ for 30 min followed by 45 cycles of PCR amplification, each consisting of 95 ℃ for 5 s and 57 ℃ for 20 s. The concentration of mRNA was calculated according to the standard curve and then normalized to that of GAPDH.

Statistical analysis

SPSS version 13.0 Software was used to perform the statistical analyses. All datas were analyzed using analysis of variance followed by a least-squares difference test. A $P < 0.05$ was used to indicate statistical significance.

RESULTS

D-lactate

Compared to the normal control, plasma D-lactate levels increased after 2 h and reached significant levels 6 h after injection in GalN/LPS-treated mice (13.57 ± 1.70 mg/L *vs* 3.76 ± 0.67 mg/L, $P < 0.01$) (Figure 1A). The levels also increased significantly 6 h after injection in GalN/TNF-α- and TNF-α-treated mice (13.02 ± 1.97 mg/L, 12.48 ± 2.49 mg/L *vs* 3.76 ± 0.67 mg/L, $P < 0.01$), but did not show an increase in LPS-, GalN- and prophylactically anti-TNF-α IgG- treated groups (6.60 ± 1.73 mg/L, 4.69 ± 1.46 mg/L, 4.50 ± 0.97 mg/L *vs* 3.76 ± 0.67 mg/L, $P > 0.05$) (Figure 1B).

Ultrastructural characteristics of the colonic mucosa

We observed obvious ultrastructural changes in the intes-

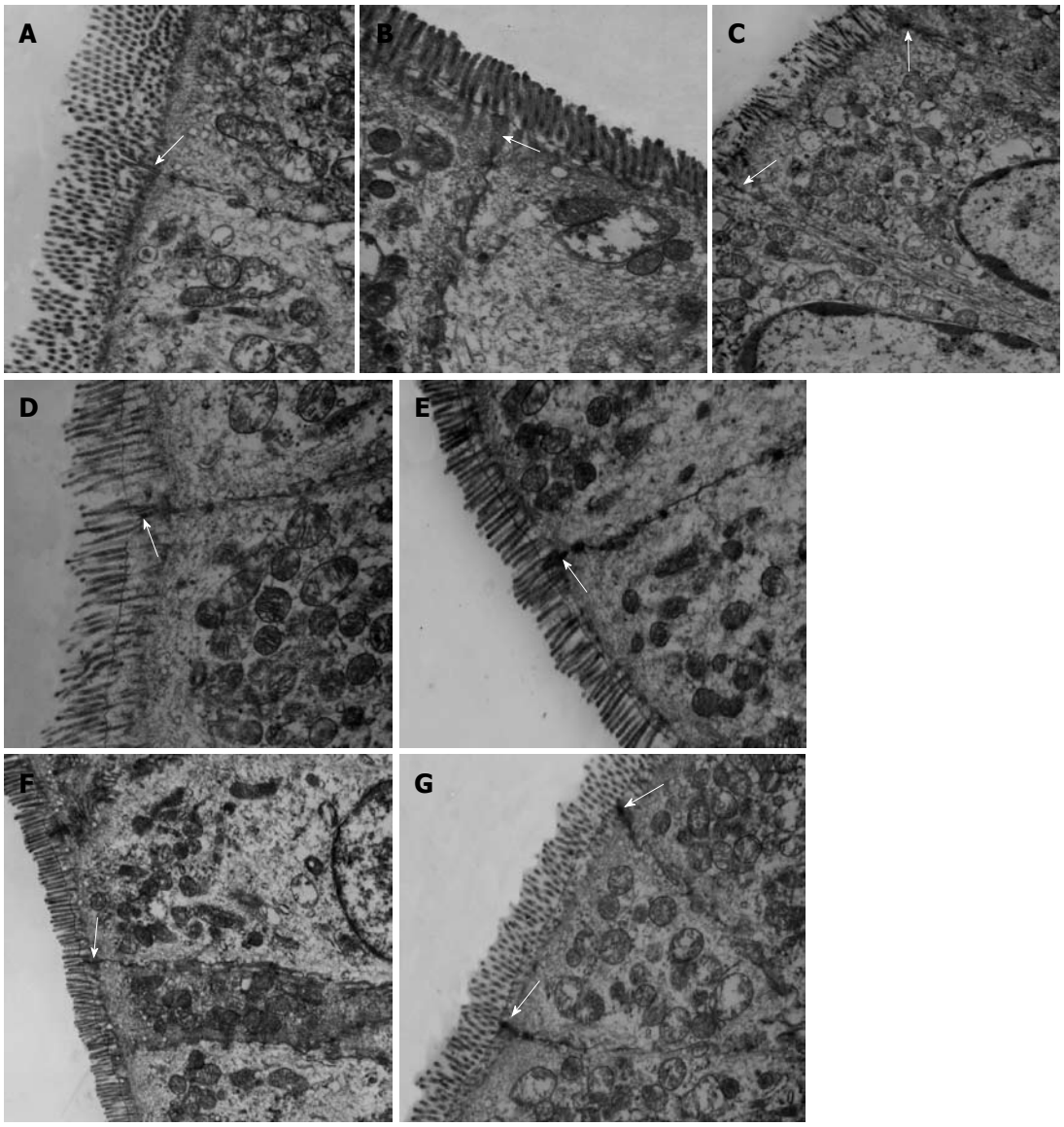


Figure 2 Transmission electron microscopy of mouse intestine (8000×). Transmission electron microscopy of mice intestine from the D-galactosamine (GalN)/lipopolysaccharide (LPS) and GalN/tumor necrosis factor alpha (TNF-α) groups (A and B), the control groups (C, D, E and F), and the group that received antibody prior to fulminant hepatic failure induction (G) (8000×). A: At 6 h after injection in the GalN/LPS group. The mitochondria of the endothelial cells were loose. Tight junctions (TJs) (arrow) were disrupted. Organelles were swollen and had reduced electron density; some microvilli were loose. A TJ (arrow) was disrupted; B: At 6 h after injection in the GalN/TNF-α group, mitochondria, organelles and microvilli were similar to A. A TJ (arrow) was disrupted; C: The GalN control group; D: The LPS control group. Epithelial cells were slightly shrunken and TJs (arrow) were intact; E: TNF-α-treated group. TJ (arrow) was intact; F: The normal saline control group. Epithelial cells and TJ (arrow) were intact; G: Anti-TNF-α antibody and the GalN/LPS group. Epithelial cells were slightly shrunken and TJs (arrows) between endothelial cells were intact.

tinal mucosa 6 h after GalN/LPS administration. Some epithelial cell microvilli were disordered and distorted, and they were sparsely distributed. The epithelial cells were swollen or shrunken. Mitochondrial swelling was observed; cristae breakdown and disrupted tight junctions were also noted. The changes in the intestinal mucosa of mice treated with GalN/TNF-α were less severe than those of mice treated with GalN/LPS. There was no disruption of TJs in the control groups, including GalN, LPS and TNF-α groups; only less swelling of epithelial cells and distorted microvilli were observed. Pathological changes in the group prophylactically treated with antibody were less severe than those in the ALF groups, and even similar to the NS control group (Figure 2).

Expression of occludin and claudin-1 proteins in the colon

Immunohistochemical analysis revealed a high density of occludin and claudin-1 expression in the lateral membrane of the intestinal epithelium in the anti-TNF-α IgG antibody-pretreated group and the saline group. The traces of occludin and claudin-1 proteins weakened in the intestinal tissue 2 h after GalN/LPS treatment, and were most pale 9 h after the injections (Figure 3A and B). The traces slightly weakened even in the TNF-α control group.

The protein level of the NS group was set to 1 as the baseline. Western blotting analysis showed that occludin and claudin-1 expressions decreased significantly in the GalN/LPS-treated mice, particularly 6 h and 9 h after

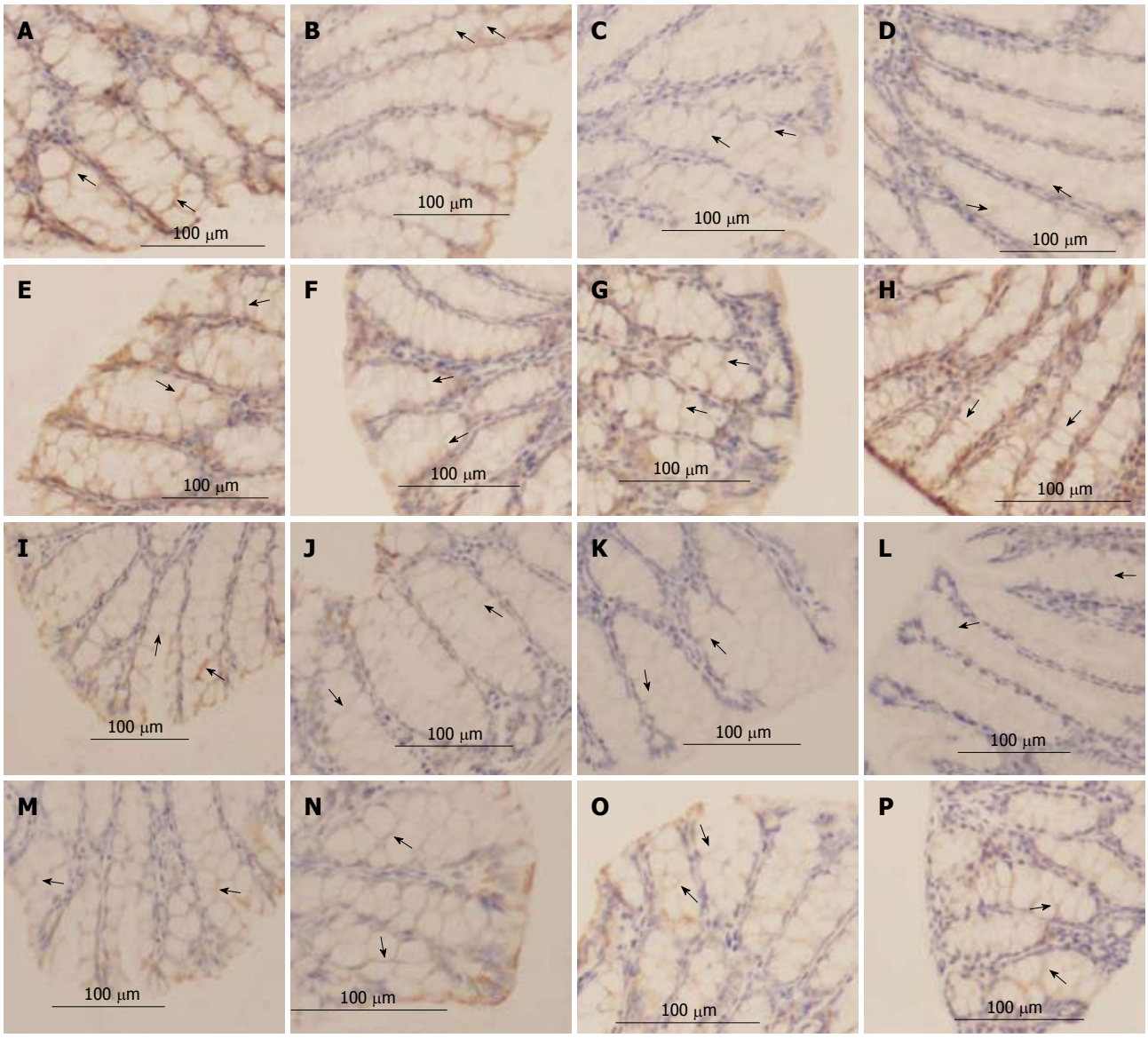


Figure 3 The tight junction proteins, occludin and claudin-1 determined by immunohistochemistry (400×). A: Occludin staining in the saline group; B-D: Occludin staining 2 h, 6 h, and 9 h after injection in the D-galactosamine (GalN)/lipopolysaccharide (LPS)-treated mice; E-H: Occludin staining 6 h after injection in the anti-tumor necrosis factor alpha (TNF-α) immunoglobulin G (IgG) antibody pretreated group within the TNF-α-treated group; the LPS-treated group and the D-GalN-treated group, respectively; I: Claudin-1 staining in the saline group; J-L: Claudin-1 staining 2, 6, and 9 h after injection in the GalN/LPS-treated mice; M-P: Claudin-1 staining 6 h after injection in the anti-TNF-α IgG antibody pretreated group, the TNF-α-treated group, the LPS-treated group and the D-GalN-treated group, respectively. The mucosal tissue sections were double-labeled for proteins (brown color). Labeled sections were analyzed immunohisto-chemically. Decreased protein staining in the epithelial cells were observed at 2-9 h after injection in the GalN/LPS-treated mice and the TNF-α-treated group, and was absent in the other controls and the antibody pretreated group.

the GalN/LPS injections (occludin: 0.6733 ± 0.0433, 0.61 ± 0.0473 fold *vs* baseline, $P < 0.01$; claudin-1: 0.8600 ± 0.0208, 0.6633 ± 0.0328 fold *vs* baseline, $P < 0.01$) (Figure 4A and C). Expressions of these proteins were decreased significantly in the GalN/TNF-α and TNF-α groups 6 h after injection (occludin: 0.6133 ± 0.0145 fold *vs* baseline, $P < 0.01$, 0.8933 ± 0.01 fold *vs* baseline, $P < 0.05$; claudin-1: 0.6267 ± 0.0145 fold *vs* baseline, $P < 0.01$, 0.92 ± 0.0173 fold *vs* baseline, $P < 0.05$), which were less in the later group (Figure 4B and D). The protein expression in the antibody-treated group was close to the normal range (occludin: 0.9467 ± 0.0285 fold *vs* baseline, $P > 0.05$; claudin-1: 0.9533 ± 0.0186 fold *vs* baseline, $P > 0.05$) (Figure 4B and D).

Expression of occludin and claudin-1 mRNA in the colon

The mRNA level of the NS group was set to 1 as baseline. We obtained a reasonable amplification curve and a standard curve of occludin, claudin-1 and GAPDH RNAs, respectively. The correlation coefficients of all standard curves were 0.999. Real-time PCR quantitative analysis showed that there were marked decreases in occludin and claudin-1 expression in ALF mice 6 h and 9 h after GalN/LPS treatment (occludin: 0.57 ± 0.159 fold *vs* baseline, 0.345 ± 0.0247 fold *vs* baseline, $P < 0.01$; claudin-1: 0.3067 ± 0.1291 fold *vs* baseline, 0.2233

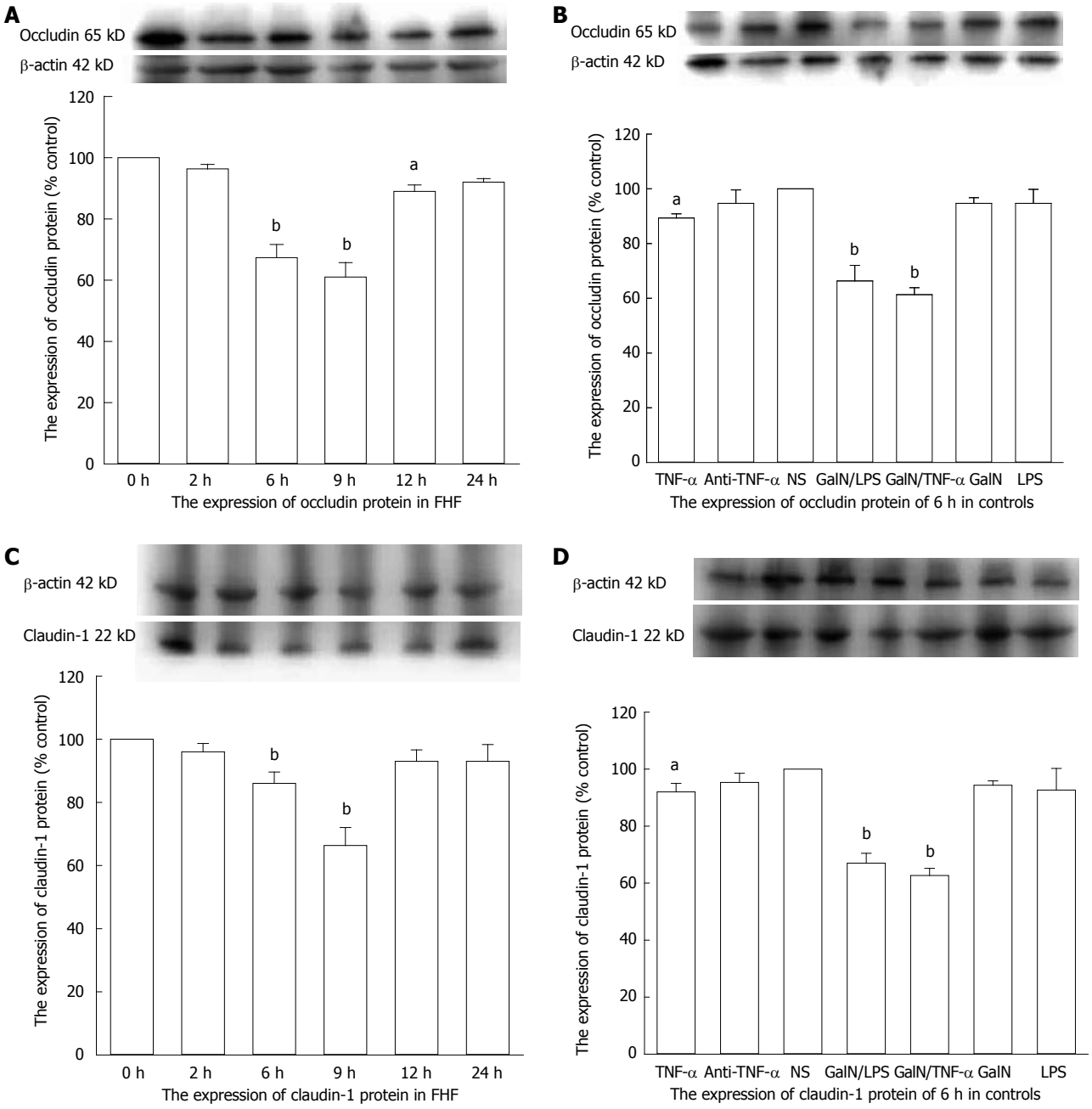


Figure 4 Mucosal colonic tissues from fulminant hepatic failure and control mice were analyzed for the protein expression of occludin and claudin-1 by Western blotting analysis. A: Occludin expression decreased significantly 6, 9 and 12 h after D-galactosamine (GalN)/lipopolysaccharide (LPS) injection; B: Occludin expression decreased significantly 6 h after injection in the GalN/ tumor necrosis factor alpha (TNF-α)- and TNF-α-treated mice; C: Claudin-1 expression decreased significantly 6 h and 9 h after GalN/LPS injection; D: Claudin-1 expression decreased significantly 6 h after injection in the GalN/TNF-α- and TNF-α-treated mice. NS: Normal saline; FHF: Fulminant hepatic failure. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* baseline. Data are reported as the mean ± SD.

± 0.1155 fold *vs* baseline, $P < 0.01$) (Figure 5A and C), as well as 6 h after GalN/TNF-α treatment and TNF-α treatment (occludin: 0.2562 ± 0.0945 fold *vs* baseline, $P < 0.01$; 0.6421 ± 0.164 fold *vs* baseline, $P < 0.05$; claudin-1: 0.1366 ± 0.0661 fold *vs* baseline, $P < 0.01$; 0.5356 ± 0.1874 fold *vs* baseline, $P < 0.05$) (Figure 5B and D). The mRNA expression in the antibody-treated group was close to the normal range (occludin: 0.8865 ± 0.0274 fold *vs* baseline, $P > 0.05$; claudin-1: 0.85 ± 0.1437 fold *vs* baseline, $P > 0.05$) (Figure 5B and D).

DISCUSSION

The intestinal mucosa is the physical and metabolic barrier that separates cytotoxic components from gut lumen. Disruption of the gastrointestinal barrier function and the diffusion of luminal toxins and pathogens into the systemic circulation are central to the pathogenesis of a number of diseases. Increased IP has been reported in many hepatic diseases[2-4,6]. IETM shows an inclination correlation with the D-lactate levels in liver cirrhosis

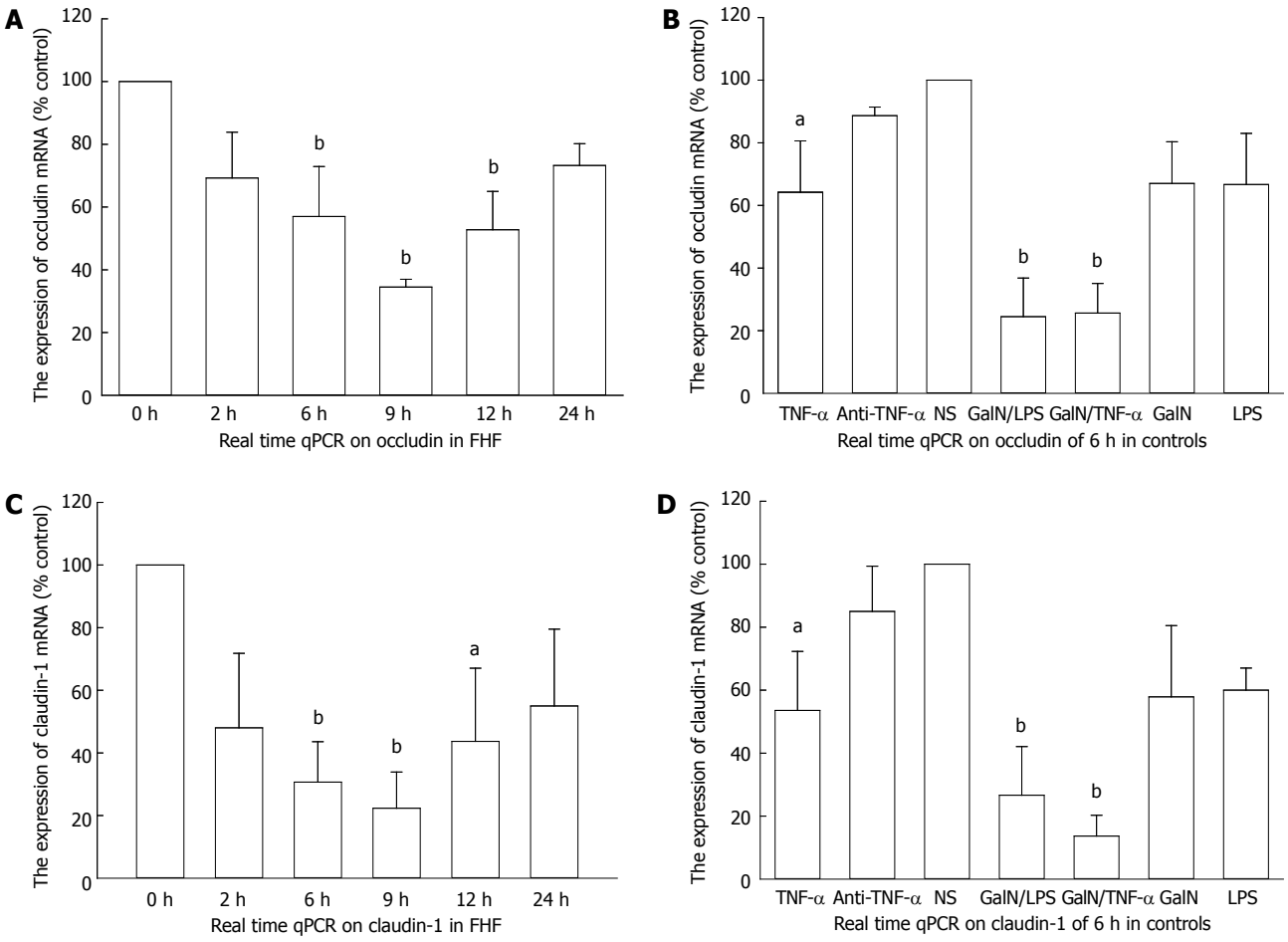


Figure 5 Real-time quantitative reverse transcription-polymerase chain reaction for occludin and claudin-1 mRNA in the fulminant hepatic failure groups and the control groups. A: Occludin significantly decreased 6 h, 9 h and 12 h after D-galactosamine (GalN)/lipopolysaccharide (LPS) injections in GalN/LPS-treated mice; B: Occludin markedly decreased 6 h after injection in the GalN/tumor necrosis factor alpha (TNF-α) or TNF-α treated mice; C: Claudin-1 significantly decreased 6 h, 9 h and 12 h after GalN/LPS injections in the GalN/LPS treated mice; D: Claudin-1 markedly decreased 6 h after injection in the GalN/TNF-α- or TNF-α-treated mice. NS: Normal saline; FHF: Fulminant hepatic failure; qPCR: Quantitative polymerase chain reaction. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* baseline. Data are reported as the mean ± SD.

and severe injuries[24,25], and appears to be related to IP. The incidence of IETM reaches 80%-100% in severe hepatitis, and plays an important role in the secondary liver injury observed in FHF[9]. How is IETM generated in FHF? When derived from overproduced bacteria in the lumen of the colon and terminal ileum[26,27], endotoxin (LPS) is translocated through the intestinal mucosa and into blood, causing IETM. Binding to LPS-binding protein, high density lipoprotein and low density lipoprotein[28,29], IETM is transported into the portal vein and achieves a higher level in the portal circulation than in peripheral veins[30]. When beyond the detoxification capability of debilitated monocytes and Kupffer cells, IETM damages hepatocytes and accelerates liver failure, even causing systemic injury[9,31]. To detect IP, we utilized D-lactate as an index. IP reached a peak 6 h post-injection of GalN/LPS. In GalN/LPS-treated mice, TNF-α levels reached its first peak 2 h post-injection[22]. When TNF-α was blocked with anti-TNF-α IgG antibody, there was a significant decrease in the IP of the mice. Considering that TNF-α causes an increase in the epithelial barrier permeability *in vivo* and *in vitro*, it is concluded that TNF-α is a crucial factor in the increased IP in FHF mice.

Borgstrom *et al*[32] have showed that at least 85% of the passive flow across the mammalian small intestine takes the paracellular route. Since the cotransporters of the apical membrane are saturated at a low nutrient concentration (below 25 mmol for glucose), but after a meal, nutrient concentrations in the proximal intestine often exceed 200 mmol, it follows that the paracellular pathway may be the major route of nutrient uptake. Drewe *et al*[33] has indicated that LPS is taken up by the intestinal mucosa predominantly by a transcellular pathway through enterocytes. Under ischemic conditions, the permeability of LPS is increased mainly by an enhanced paracellular translocation across the gut wall, whereas little apoptosis was observed in the intestinal epithelium. In several studies TNF-α induced an increase in the TJ permeability of Caco-2 cells, and did not induce apoptosis in the cells[14,34], indicating that apoptosis was not responsible for the increase in epithelial TJ permeability. In our previous study, terminal dUTP nick end labeling-positive enterocytes were not seen in the intestine of

GalN/LPS and GalN/TNF-α-treated mice until 12 h after injection[35]. The increased IP observed 6 h after GalN/LPS and GalN/TNF-α treatment was not related significantly to the transcellular pathway. Because TJ regulates the paracellular route in the leakage of macromolecules, we examined TJ with TEM and tested the protein and mRNA levels of TJ proteins. Disrupted TJ structures, down-regulated occludin and claudin-1 protein and RNA levels, and a lower density of proteins distributed in the intestinal mucosa were observed in GalN/LPS- and GalN/TNF-α-treated mice 6 h after injection. These were not observed in normal controls and in the group treated prophylactically with anti-TNF-α IgG antibody. Similar results were obtained with another TJ protein, ZO-1, in our previous study[22]. These observations indicate that TNF-α plays a central role in the regulation of TJ proteins, resulting in the disruption of TJ structure. Considering that TNF-α disrupts TJ structure and increases transepithelial permeability *in vitro* and *in vivo*, we conclude that the altered IP is induced by TNF-α *via* the destruction of TJ. The destruction of TJ by TNF-α was the result of down-regulation of TJ proteins. In the TNF-α-treated group, because of the lower extent of down-regulation of the TJ proteins, ZO-1[22], occludin and claudin-1 in the TNF-α-treated group, TJs were not visibly disrupted.

When IP was increased in GalN/LPS-treated mice 6 h after injection, the TJ structure was disrupted. In our previous study, ZO-1 showed an insignificant down-regulated expression[22], whereas occludin and claudin-1 were significantly down-regulated in GalN/LPS-treated mice 6 h after injection. This indicated a crucial role of occludin and claudin-1 in the composition of TJ. *In vitro*, TNF-α had a dose-dependent increasing effect on the permeability of the epithelium[15], and IETM had a severe effect on IP[36]. TNF-α could stimulate the expression of IL-1 and NO, which could disrupt TJ structure and increase epithelium permeability[14,18,37,38], and even synergize with interferon-α to induce intestinal epithelial barrier dysfunction[38]. *In vitro* on Caco-2 cell monolayers, TNF-α decreased the expression of phosphorylated occludin in detergent-insoluble fractions, but did not affect the expression of non-phosphorylated occludin protein. It also caused a decrease in Caco-2 transepithelial resistance and an increase in transepithelial permeability to a paracellular marker, Lucifer yellow[15]. It seemed that the combined decreased effect on IP was maximized 6 h after injection of GalN/LPS in the mouse model of FHF. Few studies on the mechanism of TNF-α-mediated decrease in the induction of TJ proteins were found. This effect might be related to nuclear factor kappa B (NF-κB). NF-κB activation and nuclear translocation of NF-κB p65 has been observed when TNF-α induces an increase in the TJ permeability of Coca-2 cell monolayers[34]. In our previous experiments on Caco-2 cell monolayers, TNF-α induced an increase of TJ permeability to Lucifer yellow, which could be partially inhibited by preincubation with 2 μg/mL anti-TNF receptor I monoclonal antibody and anti-TNF receptor Ⅱ monoclonal antibody (data not shown). This observation suggests that TNF receptor I and TNF receptor Ⅱ mediate the TNF-α induced increase in tight junction permeability. Further studies are necessary to clarify the underlying signal transduction system of the TNF-α-induced decrease in the induction of TJ proteins and the increase in TJ permeability *in vitro* and *in vivo*.

In conclusion, we found that the increase in IP stems from the downregulation of TJ proteins, especially occludin and claudin-1, and the subsequent destruction of the TJ structure that were induced by TNF-α in the mouse model of FHF.

COMMENTS

Background

Fulminant hepatic failure (FHF) is a devastating disease with many complications. Intestinal endotoxemia can lead to the pathogenesis of hepatic failure. It was therefore essential to detect the intestinal permeability (IP) in hepatic failure as the source of intestinal endotoxemia.

Research frontiers

It has been shown that altered IP occurs in many hepatic diseases. It seems that these alterations are induced by a variety of cytokines. Moreover, specified dates and underlying mechanisms deserved to illustrate about pathogenesis of IP alteration.

Innovations and breakthroughs

This is the first study to report an increased IP in FHF. Their observations support the hypothesis that tumor necrosis factor alpha down-regulates intestinal tight junction proteins and constructed to the progress of IP alteration.

Applications

The study provides insights into the mechanisms of increased IP in FHF, which probably contributes to the progress of the disease.

Peer review

This study has shown the increased IP in FHF and illustrated a probable mechanism. It therefore contributes to the understanding of intestinal complications in FHF.

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BRIEF ARTICLE

Inpatient capsule endoscopy leads to frequent incomplete small bowel examinations

Cemal Yazici, John Losurdo, Michael D Brown, Scott Oosterveen, Robert Rahimi, Ali Keshavarzian, Leila Bozorgnia, Ece Mutlu

Cemal Yazici, Department of Medicine, Mercy Hospital and Medical Center, Chicago, IL 60616, United States
John Losurdo, Michael D Brown, Ali Keshavarzian, Ece Mutlu, Division of Digestive Diseases and Nutrition, Rush University Medical Center, Chicago, IL 60612, United States
Scott Oosterveen, WellSpan Gastroenterology, York, PA 17403, United States
Robert Rahimi, Division of Digestive and Liver Diseases, University of Texas Southwestern Medical Center, Dallas, TX 75390, United States
Leila Bozorgnia, Department of Pediatrics, Los Angeles County-University of Southern California Hospital, Los Angeles, CA 9003, United States
Author contributions: Yazici C contributed to the acquisition, entry, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, and final approval of the article; Losurdo J and Brwon MD contributed to conception and design, performance of the capsule studies and data acquisition, critical revision of the article for important intellectual content and final approval of the article; Oosterveen S, Rahimi R and Bozorgnia L contributed to data acquisition and entry, revision of the article and final approval; Keshavarzian A contributed to conception and design, and final approval of the study; Mutlu E contributed to conception and design, acquisition, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, and final approval of the article.
Correspondence to: Ece Mutlu, MD, MBA, MS, Associate Professor, Division of Digestive Diseases and Nutrition, Rush University Medical Center, Chicago, IL 60612, United States. ece_mutlu@rush.edu
Telephone: +1-312-9425861 Fax: +1-312-5633883
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Abstract

AIM: To examine the predictive factors of capsule endoscopy (CE) completion rate (CECR) including the effect of inpatient and outpatient status.

METHODS: We identified 355 consecutive patients who completed CE at Rush University Medical Center between March 2003 and October 2005. Subjects for CE had either nothing by mouth or clear liquids for the afternoon and evening of the day before the procedure. CE exams were reviewed by two physicians who were unaware of the study hypotheses. After retrospective analysis, 21 cases were excluded due to capsule malfunction, prior gastric surgery, endoscopic capsule placement or insufficient data. Of the remaining 334 exams [264 out-patient (OP), 70 in-patient (IP)], CE indications, findings, location of the patients [IP *vs* OP and intensive care unit (ICU) *vs* general medical floor (GMF)] and gastrointestinal transit times were analyzed. Statistical analysis was completed using SPSS version 17 (Chicago, IL). Chi-square, *t* test or fisher exact-tests were used as appropriate. Multivariate logistic regression analysis was used to identify variables associated with incomplete CE exams.

RESULTS: The mean age for the entire study population was 54.7 years. Sixty-one percent of the study population was female, and gender was not different between IPs *vs* OPs (P = 0.07). The overall incomplete CECR was 14% in our study. Overt obscure gastrointestinal bleeding (OGB) was a significantly more common indication for the IP CE (P = 0.0001), while abdominal pain and assessment of IBD were more frequent indications for the OP CE exams (P = 0.002 and P = 0.01, respectively). Occult OGB was the most common indication and arteriovenous malformations were the most common finding both in the IPs and OPs. The capsule did not enter the small bowel (SB) in 6/70 IPs and 8/264 OPs (P = 0.04). The capsule never reached the cecum in 31.4% (22/70) of IP *vs* 9.5% (25/264) of OP examinations (P < 0.001). The mean gastric transit time (GTT) was delayed in IPs compared to OPs, 98.5 ± 139.5 min *vs* 60.4 ± 92.6 min (P = 0.008). Minimal SB transit time was significantly prolonged in the IP compared to the OP setting [IP = 275.1 ±

111.6 min *vs* OP = 244.0 ± 104.3 min (P = 0.037)]. CECR was also significantly higher in the subgroup of patients with OGB who had OP *vs* IP exams (95% *vs* 80% respectively, P = 0.001). The proportion of patients with incomplete exams was higher in the ICU (n = 7/13, 54%) as compared to the GMF (n = 15/57, 26%) (P = 0.05). There was only a single permanent SB retention case which was secondary to a previously unknown SB stricture, and the remaining incomplete SB exams were due to slow transit. Medications which affect gastrointestinal system motility were tested both individually and also in aggregate in univariate analysis in hospitalized patients (ICU and GMF) and were not predictive of incomplete capsule passage (P > 0.05). Patient location (IP *vs* OP) and GTT were independent predictors of incomplete CE exams (P < 0.001 and P = 0.008, respectively).

CONCLUSION: Incomplete CE is a multifactorial problem. Patient location and related factors such as severity of illness and sedentary status may contribute to incomplete exams.

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Key words: Capsule endoscopy; Completion rate; Inpatient; Outpatient; Hospitalization

Peer reviewers: Pietro Valdastri, MScEE, PhD, Assistant Professor of Industrial BioEngineering, The BioRobotics Institute, Scuola Superiore Sant'Anna, Polo Sant'Anna Valdera, Viale Rinaldo Piaggio, 34, 56025 Pontedera, Italy; Spiros Ladas, Professor, 1st Department of Internal Medicine-Propaedeutic, Medical School, Athens University, "Laiko" General Hospital, Agiou Thoma 17, 11527 Athens, Greece; Suryakanth Gurudu, MD, Mayo Clinic Arizona, 13400 East shea blvd, Scottsdale, AZ 85259, United States

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INTRODUCTION

In recent years, capsule endoscopy (CE) has emerged as a sophisticated and widely used tool to evaluate small bowel pathology. It has applications in Crohn's disease, celiac disease, Peutz-Jeghers syndrome, familial adenomatous polyposis, and hereditary polyposis syndromes[1-4]. CE is also particularly useful for the investigation of obscure gastrointestinal bleeding (OGB)[5,6], especially if upper and lower endoscopies fail to reveal the underlying pathology and a small bowel source is suspected. Locating the source and treating OGB has long been a challenge for physicians. Identification of the bleeding site is the key step not only to diagnose but also to provide effective treatment. During a brisk overt hemorrhage, the rapidity at which the bleeding site is found is critical. CE is therefore utilized often in patients hospitalized for bleeding and anemia. The hospital setting, however, may not be ideal in obtaining optimal results with CE. In-patients tend to be sedentary and are more prone to delayed transit or ileus, which may prolong gastric and small bowel transit times. Therefore, we hypothesized that in-patient (IP) CE is more often incomplete as compared to out-patient (OP) CE. The two previously published European studies indirectly examining this question have compared IP *vs* OP CE completion rates (CECR)[7,8], but both studies have limitations: both used a prokinetic agent and/or bowel preparation to increase CECR and neither of these interventions is part of the standard CE protocol at United States institutions. Furthermore, the patient numbers were small and the studies did not examine the effects of inpatient location (i.e., an intensive care unit (ICU) *vs* a general medical floor (GMF) setting). In this study, we aimed at overcoming these limitations by analyzing all consecutive IPs and OPs, studying a larger IP group, doing a subgroup analysis to compare the ICU and GMF settings, without the use of prokinetic and bowel preparation agents.

MATERIALS AND METHODS

This study was conducted in compliance with ethical guidelines and was approved by Rush University Institutional Review Board. All CE examinations done at Rush University Medical Center between March, 2003 and October, 2005 were identified retrospectively. A total of 355 CE exams, which were performed using a Given PillCam™ small bowel (SB) capsule, were reviewed using the Given Rapid® Access software. Patients with capsule malfunction (n = 6), prior gastric surgery (n = 3), endoscopic capsule placement (n = 3) and studies lacking sufficient data for analysis were excluded (n = 9; 8 OP, 1 IP). In the remaining 334 exams (264 OP, 70 IP), patient characteristics such as age, gender, gastric retention of the capsule, completeness of the examination (whether or not the capsule reached the cecum), gastric and small bowel transit times, indications and findings were recorded.

Subjects for capsule endoscopy had either nothing by mouth (except their medications) or clear liquids (excluding any red colored foods) for the afternoon and evening of the day before the procedure. The CE exam was delayed if subjects used iron supplementation within the past 5 d prior to the procedure.

CE exams were reviewed by two physicians. For the majority of cases, gastroenterologists who read the CE exams did not have any information regarding patient location. The hypotheses of the study were conceived after the exams were read and gastroenterologists were unaware of the study hypotheses.

Overt gastrointestinal bleed (GIB) was defined as having melena and/or hematochezia with a hemoglobin < 10 g/dL. Occult obscure GIB was defined as subjects

Table 1 Baseline characteristics of study population, capsule endoscopy indications and findings *n* (%)

Patient characteristics	IP (*n* = 70)	OP (*n* = 264)	*P* value
Age (yr)	60.9 ± 18.4	53.0 ± 17.7	0.001[1]
Gender (male/female)	34/36	96/168	0.07
Indications			
Occult obscure GIB	37 (52.9)	124 (46.9)	0.381
Overt GIB	24 (34.3)	23 (8.7)	0.0001
Abdominal pain	11 (15.7)	92 (36.7)	0.002
Assess IBD	8 (11.4)	83 (31.4)	0.001
Assess small bowel masses	1 (1.4)	17 (6.4)	0.137
Assess celiac disease	1 (1.4)	3 (1.1)	1.000
Others	0 (0)	9 (3.4)	0.213
Findings			
AVM	33 (47.1)	116 (43.9)	0.632
SB ulceration/erosion	19 (27.1)	116 (43.9)	0.011
Active bleed	11 (15.7)	12 (4.6)	0.001
Esophagitis/duodenitis/gastritis	7 (10.0)	28 (10.6)	1.000
Mass/polyp	13 (18.6)	43 (16.3)	0.649
Excess retained food	7 (10.0)	22 (8.3)	0.660
Others	6 (8.6)	19 (7.2)	0.698

IP: In-patient; OP: Out-patient; GIB: Gastrointestinal bleed; IBD: Inflammatory bowel disease; AVM: Arteriovenous malformation; SB: Small bowel. [1]*t* test; the rest, Chi-square or fisher exact test as appropriate.

who had a positive fecal occult blood test or complaints of intermittent black bowel movements, and who had both colonoscopy and upper endoscopy without any source of bleeding identified. Accurate SB transit time (SBTT) were obviously not available in those subjects in whom the capsule stopped recording before reaching the colon. In this circumstance the minimum SBTT, which reflects the minimum time the capsule spent in the small bowel, was calculated using the formula, 480 min - the gastric transit time (GTT) (min) = minimum SBTT. Four hundred and eighty minutes was used as this has the generally accepted battery life of the Given PillCam™ SB capsule at the time of the CE exams.

Statistical analysis was completed using SPSS version 17 (Chicago, IL). Chi-square, *t* test or fisher exact-test was used as appropriate. Multivariate logistic regression analysis was used to identify variables associated with incomplete CE exams. A multivariate logistic regression model was constructed using forward stepwise method with capsule reaching the cecum or not as the dependent variable using the univariate variables that were significantly predictive of incomplete capsule passage. In addition, differences in bowel transit time between the groups were compared by a survival model (Kaplan-Meier) and (Log-Rank test).

RESULTS

Study population

The baseline characteristics of the IPs *vs* OPs that underwent CE are shown in Table 1. The mean age for the entire study population was 54.7 (17-92) years. The mean age for the IPs was greater than the OPs (60.9 ± 18.4 *vs* 53 ± 17.7, $P = 0.001$). Sixty-one percents of the study population was female, and gender was not different between IPs *vs* OPs ($P = 0.07$). The indications for CE were occult OGB, overt OGB, abdominal pain, and assessment for inflammatory bowel disease (IBD), SB masses, celiac disease and other pathologies. Several patients had more than one indication for the CE examination. Occult OGB was the most common indication for both the IPs and OPs. While evaluation of overt OGB was significantly more common for the IP CE ($P = 0.0001$), abdominal pain and assessment of IBD were more frequently the indications for the OP CE exams ($P = 0.002$ and $P = 0.01$, respectively). Arteriovenous malformations (AVM) were the most common finding both in the IPs and OPs. Active bleeding was seen more often in the IPs ($P = 0.001$); while SB ulcerations and erosions were more frequently observed in the OP CE exams ($P = 0.011$).

Completion rates of CE

The overall incomplete CECR was 14% in our study. A higher number of patients in the OP group had complete CE with visualization of the entire SB, compared to the IP group (90.5% *vs* 68.6%, $P < 0.001$). The capsule remained within the stomach for the duration of the study in 8.6% (6/70) of the IPs and 3% (8/264) of the OPs ($P = 0.04$). When these cases of gastric capsule retention were excluded, 25% (16/64) of the IPs and 6.6% (17/256) of the OPs had exams that were not complete, as the capsule did not reach the cecum ($P = 0.001$). Therefore, both gastric capsule retention with reduced entry into the SB as well as delayed transit in the SB played a role in the incomplete CE exams in the IPs. Obstructing lesions in the SB were uncommon. There was only a single permanent small bowel retention case in the dataset, who was an OP with a previously unrecognized stricture. The remainder of the incomplete SB exams appeared to be due purely to slow transit. To exclude a diagnosis related bias in the analysis, we also looked at the OGB subset of our data. CECR was also significantly higher in OGB patients who had OP *vs* IP exams (95% *vs* 80% respectively, $P = 0.001$). Therefore, the result obtained can not be solely explained by differences in the OP *vs* IP diagnoses.

Transit times in the stomach and small bowel

A Kaplan-Meier plot of total bowel transit time for the IPs *vs* the OPs is shown in Figure 1. As time passes more studies get completed in the OP group compared to the IP group. The median total capsule transit time to the cecum was longer in IP *vs* OP (368 min *vs* 279 min) ($P < 0.0001$, log-rank for median total capsule transit time).

In 10 patients (3 IPs and 7 OPs), GTT were not recorded into the CE report. In the remaining 324 patients, the mean GTT was prolonged in the IP setting. GTT was 98.5 ± 139.5 min in IP *vs* 60.4 ± 92.6 min in OP setting ($P = 0.008$). After removing those patients with gastric capsule retention and lack of GTT measurements from the dataset, minimum SBTT were signifi-

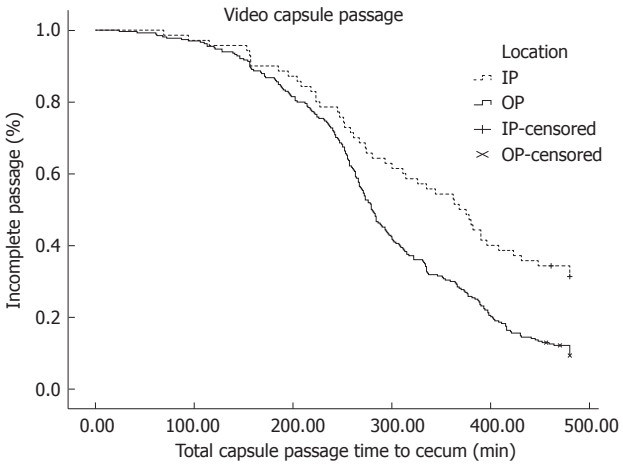


Figure 1 Kaplan-Meier plot of total capsule passage time to cecum. Incomplete passage percentage and total capsule passage time to cecum were different between inpatients (IP) and outpatients (OP). Data is shown for the IP group (dotted line) *vs* the OP group (solid line). X-axis represents bowel passage time and Y-axis represents the percentage of patients in whom the capsule has not reached the cecum.

cantly prolonged in the IP setting (275.1 ± 111.6 min) *vs* the OP setting (244.0 ± 104.3 min) ($P = 0.037$).

Effect of inpatient location

Among the 70 IPs studied, 13 were in the ICU and 57 were on a GMF. There was no statistical difference in mean age and gender in the ICU *vs* GMF groups, although the ICU patients were relatively older (68.8 ± 13.6 years *vs* 59.1 ± 19.0 years, $P = 0.084$).

The proportion of patients with incomplete exams was higher in the ICU ($n = 7/13$, 54%) as compared to the GMF ($n = 15/57$, 26%) ($P = 0.05$). Gastric capsule retention also was more common among ICU patients compared to the GMF [2/13 (15.3%) *vs* 4/57 (7%), $P = 0.331$]. When subjects with gastric retention were excluded, the proportion of subjects with incomplete small bowel exams were numerically higher 5/11 (45%) in the ICU setting *vs* 11/53 (32%) in the GMF setting, but the difference was not statistically significant ($P = 0.085$). The GTTs were not significantly different between the ICU and GMF patients (154.4 ± 181.4 min *vs* 85.0 ± 125.8 min, $P = 0.108$) although a trend toward longer GTT in ICU patients was noted. Minimum SBTT were also not significantly different between the ICU (272.1 ± 131.4 min) and GMF patients (275.8 ± 108.4 min) ($P = 0.922$).

Risk factors for an incomplete exam

We looked at the effects of potential risk factors for an incomplete exam in univariate analyses in all subjects (Table 2). Among pre-procedure risk factors, location (IP *vs* OP setting) and having an indication for gastrointestinal (GI) bleed (either occult or overt) were predictive of incomplete capsule passage. Among post-procedure risk factors, the following findings were predictive of having an incomplete exam: having an AVM, blood, visible vessel, lymphoid hyperplasia, gastric vascular ectesia, findings suggesting Crohn's disease (CD), excess food particles in the GI tract, phlebectasia, having large gastric folds, white appearing villi, GTT, and SBTT. We could not evaluate the effect of medication use in the entire dataset due to lack of accurate medication data in outpatients.

Table 2 Identification of potential risk factors for an incomplete exam by univariate analysis in all patients

Pre-procedure factors	*P* value	Post-procedure factors	*P* value
Location	0.0001	AVM	0.001
Occult GIB	0.042	Blood	0.016
Overt GIB	0.054	Visible vessel	0.013
Abdominal pain	0.608	Lymphoid hyperplasia	0.014
Assessment of IBD	0.142	GAVE	0.051
History of FAP	0.999	Findings suggestive of CD	0.007
History of DA	0.199	Excess food	0.006
Nausea/vomiting	0.879	Phlebectasia	0.011
Diarrhea	0.929	Large gastric folds	0.010
Colon polyps	0.999	White villi	0.01
History of OVR	1	Edema	0.415
History of carcinoid tumors	0.999	Submucosal mass	0.372
History of PJS	1	Mucosal mass	0.119
History of VE	1	Erosions	0.350
Age	0.113	Ulcer	0.308
Gender	0.232	Duodenitis	0.597
		Diverticulitis	0.461
		Mucosal break	0.078
		Esophagitis	0.062
		Stricture	0.399
		Reader	0.982
		GTT	0.0001
		SBTT	0.001

GIB: Gastrointestinal bleed; IBD: Inflammatory bowel disease; FAP: Familial adenomatous polyposis; DA: Duodenal adenoma; OVR: Osler Veber Rendu; PJS: Peutz Jeghers syndrome; VE: Vascular ectasia; AVM: Arteriovenous malformation; GAVE: Gastric vascular ectasia; CD: Crohn's disease; GTT: Gastric transit time; SBTT: Small bowel transit time.

Table 3 Effect of medication use and comorbidities on capsule endoscopy completion rate in hospitalized patients

Risk factors	*P* values
Diabetes	0.416
Neuropathy	0.382
Hypotension	0.947
Immobility	0.381
Medications in aggregate	0.271
Narcotics	0.311
Anticholinergics	0.815
Beta-blockers	0.711
Calcium channel blockers	0.931
Inotropes	0.197
Motility enhancers	0.337

Medications which affect GI system motility were tested both individually and also in aggregate in univariate analysis in hospitalized patients (Table 3). These medications included narcotics, anticholinergics, beta-blockers, calcium channel blockers, inotropic agents, and motility enhancers. None of the medications individually or in aggregate were predictive of "capsule passage" or

Table 4 Independent predictors of incomplete capsule endoscopy in multivariate analysis

Factors	Beta value	SE	Wald × 2	*P* value	Odds ratio	95% CI
Intercept	-3.369	0.349	93.344	0.000		
Location	1.457	0.415	12.328	0.000	4.294	1.904-9.686
GTT	0.012	0.003	18.177	0.000	1.012	1.006-1.017

GTT: Gastric transit time.

"incomplete capsule exam" ($P > 0.05$, Table 3). Other factors such as diabetes, neuropathy, hypotension, and immobility were not predictive of "capsule passage" or "incomplete capsule exam" either ($P > 0.05$, Table 3).

In the multivariate logistic regression model that utilized all patients, except those who did not have gastric transit times recorded, patient location (IP *vs* OP) and GTT were independent predictors of incomplete CE exams ($P < 0.001$ and $P = 0.008$, respectively). Further details of the model are given in Table 4.

DISCUSSION

Our study represents a first look at the CECR in IPs in the United States, and demonstrates that both GTT and SBTT are significantly prolonged in IP CE exams compared to OP exams. In a multivariate regression model, we show that patient location (IP *vs* OP) and GTT are independent predictors of incomplete CE, suggesting factors other than GTT must be contributing to the lower CECR in inpatients. Here we demonstrate that increased SBTT is another important contributor for low CECR in addition to GTT. We further show that the CECR is lowest in the ICU setting.

CECR in patients with OGB has been investigated previously. In these studies, CECR are variable and lower than what we note in our study. In a prospective European study of only mild to moderate obscure overt GI bleeders, incomplete IP CEs were 21.6%, but in 75% of the incomplete exams there was some localization of the bleed site[9]. However, this study excluded all severe bleeders and inpatients with other indications. In another study of 48 inpatient obscure overt GI bleeders from Europe, incomplete CE rates were 27%[10]. In a recent Canadian study of 535 patients, overt GI bleed was found to be a major risk factor for incomplete CE ($P = 0.002$, OR = 2.7) and the overall incomplete CE rate was 29.5%[11]. However, in this study, only 31 subjects were inpatients. Therefore, hospitalization status did not reach statistical significance despite a large trend ($P = 0.054$). In studies that analyzed CECR in patients with GIB, CECR in relation to patient location was not examined and direct comparisons to outpatients were not complete. Using subgroup analysis of our patients with GIB, we found a lower IP CECR compared to these prior studies (95% in OP *vs* 80% in IP). More interestingly, regardless of the GIB diagnosis, incomplete CE rate was also significantly lower in the OP setting compared to the IP setting ($P = 0.001$).

The relationship between CECR and hospitalization was addressed more directly in two other previous European studies. When all indications were taken into account, incomplete CE rate was higher in hospitalized patients, 34%-35%[7,8]. Our results in the United States confirm these findings in a larger dataset. GTT was elevated in the IP setting in both studies, similar to our findings. In the former studies, there were additional interventions to improve CE quality: per protocol, some patients received bowel preparation[8], while others received both bowel preparation and prokinetic agents[7]. Bowel preparation and the use of prokinetic agents are not routine in the United States. None of these prior studies specifically investigated the effect of IP location in CECR, and in one of these studies, patients underwent a 23 h hospitalization solely to perform the CE, which is not done in the United States. In addition lower IP CECR shown in these studies were attributed to decreased gastric motility; whereas we now show that the minimum SBTTs are also significantly prolonged in patients who had IP CE, suggesting that factors such as generalized dysmotility of gastrointestinal tract may be at play.

The overall CECR has been reported to be 81.1% and 83.5% in two recent meta-analyses[12,13]. The first meta-analysis compared CECR in patients who were prepped with purgatives *vs* clear liquid diet prior to the CE and showed that there was no significant difference in CECR, although the purgative use resulted in better small bowel visualization quality and increased diagnostic yield[13]. The second study reported an overall CECR of 83.5% in patients with OGIB, CD, and neoplastic lesions[12]. The overall CECR in our study at 86% reflects a slightly higher value than reported in these two meta-analyses. Nevertheless, even if we were to assume that the CECR in our OP group was as low as 81%-83% (similar to the two meta-analyses), this would still be markedly numerically higher than our observed CECR of 68% in the IPs, and should not alter our conclusions. Our CECR may perhaps be reflective of the careful case selection at a tertiary urban medical center, in order to avoid retention. Alternative explanations could include a larger number of OPs compared to IPs in our study. Notably, the percentage of OPs and IPs were not delineated in the meta-analyses. While the latter studies included in the meta-analyses all come from the English literature, they could be conducted in various countries, where CE may not be routinely done as an outpatient. Additionally even within the United States, the capture area of each medical center may be different and may require some subjects to be IPs especially if the patient catchment area is wide and mostly rural. Our findings of lower CECR rates in the ICU setting might suggest that the severity of the acute illness may also play important roles in CECR. However, possible confounding factors such as medication use and certain comorbidities such as diabetes, neuropathy, and hypotension were not predic-

tive of incomplete CE in hospitalized patients. This suggests that multiple factors may have a combined effect in the outcomes observed in hospitalized patients. While our hospitalized patients were older than our outpatients and while our ICU patients were older than our GMF patients, age was not an independent factor that explained CECR differences, which have previously been demonstrated[10,14].

Our study has several strengths including a larger IP sample size and a sub-group analysis on patient location and GIB. In our study, no confounding prokinetic agents or bowel preparations were used, and the hospitalization status was exclusively based on the severity of patients' clinical condition reflective of the current practice patterns in the United States. The major limitation of our study is its retrospective design; therefore, further prospective studies in inpatients are necessary to confirm our observations. Additionally, we took consecutive patients who had CE, and did not do a matched case-control design. This resulted in more subjects in our OP group compared to IP group - due to increasing use of CE in the OP setting. Therefore, it is possible that having less number of IPs may have resulted in potential unidentified confounders. As such, larger studies on IPs are needed to confirm our findings. Another potential limitation of our study is that, the readers of CE examinations were not blinded to the patient's clinical data. However, the readers were blinded to the hypotheses, making an outcome bias less likely to impact the study results. The lack of data regarding medication use, diabetes, and neuropathy in the outpatient group, lack of a generalized quality measure of the views obtained during CE exams, and the single center design are other limitations of our study.

Several interventions have been undertaken to improve CECR. The results of the studies which investigated the effect of bowel preparation on CECR were not consistent with each other[15-22], but improved image quality has been reported. Previous studies also investigated the role of endoscopic placement of capsule and/or real-time viewing and showed that these approaches can improve CECR[23-26]. Use of 12 h CEs could also be used to improve CECR[27]. Future studies are needed to examine the effects of such interventions to improve CECR among inpatients.

In conclusion, inpatients have a lower than usual CECR, which is more pronounced if admitted to the ICU. While gastric retention is a reason in some patients for the lower CECR, there seems to be generalized dysmotility in inpatients with incomplete CE exams. Other factors embodied in the IP setting such as sedentary status and acuity of the illness could be partially contributing. A large prospective study is needed to further delineate the most important factors that contribute to incomplete exams in the inpatient setting and determine the impact of incomplete CE on patient outcomes.

COMMENTS

Background

Capsule endoscopy (CE) is being used widely to evaluate small bowel pathologies. It has applications in Crohn's disease, celiac disease, Peutz-Jeghers syndrome, familial adenomatous polyposis, hereditary polyposis syndromes, and particularly in the investigation of obscure gastrointestinal bleeding. However, the hospital setting may not be ideal in obtaining optimal results with CE as hospitalized patients tend to be more sedentary and have prolonged gastric and small bowel transit times.

Research frontiers

Two previously published studies indirectly examined the effect of patient location on CE completion rates (CECR), but both studies had limitations. Earlier studies also investigated the effect of bowel preparation, endoscopic placement of capsule and/or real-time viewing, and the use of 12 h CEs on CECR.

Innovations and breakthroughs

This is the first study to investigate the effect of patient location on CECR in United States. The authors demonstrated that both gastric transit time (GTT) and small bowel transit time (SBTT) are significantly prolonged in in-patient (IP) CE exams compared to out-patient (OP) exams. The authors also showed that patient location (IP *vs* OP) and GTT are independent predictors of incomplete CE. The authors further show that the CECR is lowest in the intensive care unit setting. While gastric retention is a reason in some patients for the lower CECR, there seems to be generalized dysmotility in IPs with incomplete CE exams. Other factors such as sedentary status and acuity of the illness could be partially contributing to lower CECR in hospitalized patients.

Applications

In addition to patient location other factors such as severity of illness and sedentary status may contribute to incomplete exams in hospitalized patients. CECR can be improved by doing CE studies as outpatient, placing the capsule endoscopically, using real-time viewing or 12 h CEs, and limiting the use of medications which decrease gastrointestinal transit time.

Terminology

CE is a procedure in which a small camera (capsule) swallowed to investigate the small bowel pathologies; CECR shows the percentage of CE exams that are completed; GTT is the time capsule spends in the stomach during the study; SBTT is the time capsule spends in the small bowel during the study.

Peer review

In this study, the authors investigated the effect of IP and OP status on SBTT and CECR. This is an interesting and well written paper.

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BRIEF ARTICLE

Crohn's disease genotypes of patients in remission *vs* relapses after infliximab discontinuation

Cathy Lu, Alistair Waugh, Robert J Bailey, Raeleen Cherry, Levinus A Dieleman, Leah Gramlich, Kata Matic, Mario Millan, Karen I Kroeker, Daniel Sadowski, Christopher W Teshima, Dennis Todoruk, Clarence Wong, Karen Wong, Richard N Fedorak

Cathy Lu, Alistair Waugh, Levinus A Dieleman, Karen I Kroeker, Christopher W Teshima, Karen Wong, Richard N Fedorak, Division of Gastroenterology, Zeidler Ledcor Center, University of Alberta, Edmonton, AB T6G 2X8, Canada
Robert J Bailey, Leah Gramlich, Kata Matic, Daniel Sadowski, Dennis Todoruk, Clarence Wong, Division of Gastroenterology, Royal Alexandra Hospital, Edmonton, AB T5H 3V9, Canada
Raeleen Cherry, Division of Gastroenterology, Grey Nuns Hospital, Edmonton, AB T6L 5X8, Canada
Mario Millan, Division of Gastroenterology, Misericordia Hospital, Edmonton, AB T5R 4H5, Canada
Author contributions: Lu C collected and analyzed data and drafted manuscript; Waugh A contributed to the collection of the data; Bailey RJ, Cherry R, Dieleman LA, Gramlich L, Matic K, Millan M, Kroeker KI, Sadowski D, Teshima CW, Todoruk D, Wong C and Wong K contributed to revision of manuscript; Fedorak RN contributed by developing study concept and design, interpretation of data, and critical revision of manuscript.
Supported by Center of Excellence for Gastrointestinal, Inflammation and Immunity Research at the University of Alberta
Correspondence to: Richard N Fedorak, MD, Division of Gastroenterology, Zeidler Ledcor Center, University of Alberta, Edmonton, AB T6G 2X8, Canada. richard.fedorak@ualberta.ca
Telephone: +1-780-4926941 Fax: +1-780-4928121
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Abstract

AIM: To investigate genetic differences between Crohn's disease (CD) patients with a sustained remission *vs* relapsers after discontinuing infliximab while in corticosteroid-free remission.

METHODS: Forty-eight CD patients received infliximab and were in full corticosteroid-free clinical remission but then discontinued infliximab for reasons other than a loss of response, were identified by review of an electronic database and charts. Infliximab-associated remission was defined as corticosteroid-free plus normalization of clinical disease activity [CD activity index (CDAI) < 150] during follow-up visits based on physician global assessments. A CD relapse (loss of infliximab-induced remission) was clinically defined as a physician visit for symptoms of disease activity (CDAI > 220) and a therapeutic intervention with CD medication(s), or a hospitalization with complications related to active CD. Genetic analyses were performed on samples from 14 patients (n = 6 who had a sustained long term remission after stopping infliximab, n = 8 who rapidly relapsed after stopping infliximab). Nucleotide-binding oligomerization domain 2 (NOD2)/caspase activation recruitment domain 15 (CARD15) polymorphisms (R702W, G908R and L1007fs) and the inflammatory bowel disease 5 (IBD5) polymorphisms (IGR2060a1 and IGR3081a1) were analyzed in each group.

RESULTS: Five single nucleotide polymorphisms of IBD5 and NOD2/CARD15 genes were successfully analyzed for all 14 subjects. There was no significant increase in frequency of the NOD2/CARD15 polymorphisms (R702W, G908R and L1007fs) and the IBD5 polymorphisms (IGR2060a1 and IGR3081a1) in either group of patients; those whose disease relapsed rapidly or those who remained in sustained long term remission following the discontinuation of infliximab. Nearly a third of patients in full clinical remission who stopped infliximab for reasons other than loss of response remained in sustained clinical remission, while two-thirds relapsed rapidly. There was a marked difference in the duration of clinical remission following discontinuance of infliximab between the two groups. The patients who lost remission did so after 1.0 years ± 0.6 years, while those still in remission were at the time of this study, 8.1 years ± 2.6 years post-discontinuation of infliximab, P < 0.001. The 8 patients who had lost remission after discontinuing infliximab had a

mean number of 5 infusions (range 3-7), with a mean treatment time of 7.2 mo (range 1.5 mo-15 mo). The mean duration of time from the last infusion of infliximab to the time of loss of remission was 382 d (range 20 d-701 d). The 6 patients who remained in remission after discontinuing infliximab had a mean number of 6 infusions (range 3-12), with a mean treatment duration of 12 mo (range 3.6 mo-32 mo) (P = 0.45 relative to those who lost remission).

CONCLUSION: There are no IBD5 or NOD2/CARD15 mutations that predict which patients might have sustained remission and which will relapse rapidly after stopping infliximab.

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Key words: Infliximab; Anti-tumor necrosis factor alpha; Crohn's disease; Inflammatory bowel disease; Genotype

Peer reviewers: Stefanos Karagiannis, MD, PhD, Gastrointestinal and Liver Unit of University of Athens, General and Oncology Kifissia Hospital "Agioi Anargyroi", 14564 Kaliftaki, Kifissia, Greece; Bruno Bonaz, MD, PhD, Clinique Universitaire d'Hépato-Gastroentérologie, CHU de Grenoble, BP 217, 38043 Grenoble Cedex 09, France

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INTRODUCTION

Crohn's disease (CD), a type of inflammatory bowel disease (IBD), is a complex chronic condition characterized by relapsing transmural inflammation throughout the gastrointestinal tract. The etiology of CD remains unclear and as with most complex-trait diseases, the development of IBD involves a combination of genetic and environmental risk factors. Epidemiological data and family studies do suggest a genetic basis for CD[1,2]. For instance, the single greatest risk factor for developing IBD is having an affected family member, as demonstrated by twin studies in which a monozygotic twin of a CD proband has approximately a 1 in 3 lifetime risk of developing IBD[3]. Genome-wide association studies have found approximately 100 genetic loci to be significantly associated with IBD[4].

In terms of CD itself, a potent proinflammatory cytokine, tumor necrosis factor alpha (TNF-α), is a well-known mediator of inflammation[5]. Clinical trials have shown anti-TNF-α agents such as infliximab to be highly efficacious in terms of induction and maintenance responses (ACCENT 1 and 2)[6,7]. However, anti-TNF-α agents are costly, and the long-term safety risks such as development of autoantibodies, drug-induced lupus, infection, and neoplasia can be concerning in the long term[5,8-10]. Furthermore, the appropriate duration of anti-TNF-α therapy for CD patients who are in remission is as yet undefined; specifically, the factors which affect the duration of sustained clinical benefit after discontinuation of infliximab remain poorly understood.

As part of a project to achieve such an understanding, a longitudinal cohort study at our institution of over 7 years duration, identified CD patients who were in full corticosteroid-free clinical remission and then discontinued infliximab for reasons unrelated to loss of response[11]. This study demonstrated that one third of these CD patients remained in a sustained clinical remission for up to 7 years after the infliximab was discontinued, and that concomitant drug therapy, number of infliximab doses, age, gender, and CD location were not predictive of this sustained clinical benefit. This finding lends support to the hypothesis that a patient-unique genotype of CD may play a role in determining the duration of long-term remission after the discontinuation of anti-TNF-α agent therapy.

Research on CD genotypes associated with anti-TNF-α therapy has generally studied infliximab responders and compared the results to infliximab non-responders. Caspase activation recruitment domain 15 (*CARD15*) was the first gene to be associated with CD[12,13]. The nucleotide-binding oligomerization domain 2 (*NOD2*)/*CARD15* product is an intracellular protein involved in recognizing bacterial lipopolysaccharides in the nuclear factor kappa B-TNF-α pathway[14]. This *NOD2*/*CARD15* gene is known to be associated with susceptibility to CD[12,15]. Previous studies have identified certain TNF-α polymorphisms as predictive of a lack of response to infliximab[16], while other studies focusing on *NOD2*/*CARD15* mutations (R702W, G908R, 1007fs)[17,18] have not confirmed a relationship between such mutations and the response to anti-TNF-α agents. Urcelay *et al*[19] studied 40 Spanish CD patients who received infliximab, 25 responders and 15 non-responders, and identified a homozygous mutant in the IBD5 region on chromosome 5q31 that was significantly associated with a lack of response to infliximab[19]. However, no studies have reported correlations of CD genotypes for patients who relapse rapidly versus patients who do not relapse after discontinuing anti-TNF-α therapy.

This study thus examined a cohort of CD patients who had discontinued infliximab for reasons other than non-response while in full corticosteroid-free remission, to determine if genotypic differences might contribute to the varying lengths of post-infliximab remission times observed.

MATERIALS AND METHODS

Selection of infliximab-treated patients in remission who then discontinued infliximab

This study was reviewed and approved by the ethics

board at the University of Alberta prior to study initiation and subjects were provided informed consent before study participation. Forty-eight Caucasian CD patients who received infliximab between July 2001 and July 2007, and who were in full corticosteroid-free clinical remission but then discontinued infliximab for reasons other than a loss of response, were identified by review of an electronic database and chart reviews. Reasons for infliximab discontinuance included physician choice, loss of insurance coverage, fear of side-effects, adverse events, and pregnancy. These patients were referred from the IBD practices of thirteen gastroenterologists associated with the University of Alberta. Long-term follow-up data for each patient was available through to July 6, 2011.

Patients with CD were eligible for study inclusion based on the following criteria: (1) clinical response to infliximab (5 mg/kg) induction dosing administered at weeks 0, 2 and 6; (2) advancement to scheduled maintenance infliximab therapy every 8 wk; (3) maintenance of a stable corticosteroid-free clinical benefit for at least 6 mo; (4) discontinuation of infliximab therapy for reasons other than loss of response; and (5) sufficient follow-up that allowed assessment of ongoing wellness and/or disease relapse. A subject was excluded from the study if he/she was under the age of 18 years. Eligible patients were contacted via telephone by a research associate with a request for participation in the current study.

Standardized infliximab administration

Each patient included in the study had received her or his infliximab in accordance with the Canadian Association of Gastroenterology's anti-TNF-α treatment guidelines for CD patients[20]. The "step-up" approach specified by these guidelines requires that both corticosteroids and immunosuppressive (azathioprine or methotrexate) treatments have failed the patient due to a lack of response or intolerance before infliximab therapy is begun.

Definition of infliximab-induced remission

Infliximab-associated induction and maintenance remission were defined as corticosteroid-free plus normalization of clinical disease activity [CD activity index (CDAI) < 150] during follow-up based on physician global assessments at each successive visit. The dates of each initial and final infliximab infusion were determined using an infliximab infusion database.

Definition of loss of remission after discontinuing infliximab

A CD relapse, and thus loss of infliximab-induced remission (CDAI > 220), for those patients who had discontinued infliximab was defined as a physician or hospital visit for documented symptoms of disease activity and a therapeutic intervention with CD medication(s), or a hospitalization with complications related to active CD. The date of a CD relapse after discontinuation of infliximab therapy was identified through review of patient medical records. Mucosal healing, laboratory investigations such as CRP and radiological evaluation were analyzed for each patient but not reported in this paper.

Genotyping

Informed consent for participation in the study and genotyping was obtained from those participants who met the inclusion criteria and who were willing to join the study. Urban patients attended an appointment at the University of Alberta outpatient gastroenterology clinic, where an 8 mL blood sample was obtained via arm venipuncture. For patients residing in rural areas, the blood sample was obtained by the patient's local laboratory and was couriered to the University of Alberta hospital for genetic processing.

The laboratory scientists who analyzed the disease loci from these samples were blinded to the study groups as they performed the following steps. Genomic DNA was purified from peripheral blood leukocytes. All sam-ples were amplified from an individual FTA card. Five single nucleotide polymorphisms (SNPs) (R702W, G908R, L1007fs, IGR2060a1, IGR3081a1) were detected. R702W and G908R were analyzed using the SNaPshot method (Single base pair extension). L1007fs, IGR2060a1, and IGR3081a1 were analyzed using the Sanger sequencing method. All samples were run on a capillary instrument. Previously sequenced samples were used as controls. The polymerase chain reaction primers are listed in Table 1.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (Statistical Package for the Social Sciences Inc., Chicago, IL). Association between genotype mutation and CD remission status was estimated using the odds ratio with a 95% CI. Fisher's exact test was utilized to calculate *P* values. A *P* value of 0.05 was considered to indicate statistical significance.

RESULTS

Patient recruitment

A total of 48 CD patients whose disease was in an infliximab-induced corticosteroid-free clinical remission, and who had then discontinued infliximab for reasons other than non-response (see Methods above) were identified from an electronic database and chart reviews. Six patients were excluded from the study for the following reasons: 2 patients were excluded due to non-Caucasian ancestry and its possible confounding effects on genetics and 4 patients were not interested in participating. Of the 42 patients who met the inclusion criteria, 3 had changed their telephone number and were unable to be contacted, 5 had moved out of province, 10 rural patients agreed to participate but did not have blood samples drawn, 3 patients missed venipuncture appointments, and 7 could not be reached by telephone despite two messages being left. The remaining fourteen CD patients were stratified into a group of those still in full clinical remission (n = 6) and a group of those who had

Table 1 Polymerase chain reaction primers used for genotyping

Genetic variant	Sense primers	Antisense primers
Arg702Trp	5′-CAT CTG AGA AGG CCC TGC TC(C/T)-3′	5′-CAG ACA CCA GCG GGC ACA-3′
Gly908Arg	5′-TTG GCC TTT TCA GAT TCT GG(G/C)-3′	5′-CCC CTC GTC ACC CAC TCT G-3′
Leu1007fs insC	Not available	Not available
IGR2060a1	5′-CTC ATT ACA TCC TTG CAA CCC T(G/C)-3′	5′-GAC ACA TGG TGT GAG CTC AGT CA-3′
IGR3081a1	5′-TCG CGT GAG TCC TAT TCT TTC T(T/G)-3′	5′-TTC ATA CTT CCA GCA GCG GG-3′

Table 2 Characteristics for Crohn's disease patients still in remission and those who lost remission after discontinuing infliximab

Characteristic	Remaining in remission following discontinuing infliximab (n = 6)	Lost remission following discontinuing infliximab (n = 8)
Male:female (n)	4:2	4:4
Age of Crohn's disease onset (yr), mean (range)	30 (14-47)	26 (15-38)
Current age (yr), mean (range)	53 (41-60)	41 (25-57)
Duration of disease prior to first infliximab infusion (yr), mean ± SD	15 ± 8.0	10.1 ± 11.3
Age at first infliximab infusion (yr), mean ± SD	45 ± 7.6	36 ± 11.1
Number of infusions, mean ± SD	5.7 ± 3.5	4.7 ± 1.7
Duration of infliximab treatment (mo), mean (range)	12 (3.6-36.2)	7.2 (1.5-15)
Duration of remission after infliximab stopped (yr), mean ± SD	8.1 ± 2.6[1]	1.0 ± 0.6[2]
Disease distribution, n (%)		
Ileum	2 (33)	2 (25)
Ileo-colonic	3 (50)	4 (50)
Colon	1 (17)	2 (25)
Current medications in patients who lost remission, n		
Adalimumab		4
Infliximab		2
Azathioprine alone		1
No Biological or immunosuppressive therapy[3]		1

[1]All patients still remain in remission at the time of this study; [2]Follow-up time from last dose of infliximab to either date of flare or last day of study (July 6, 2011), $P < 0.001$; [3]This patient developed both basal cell and renal cell carcinoma.

lost remission (n = 8).

Characteristics of patients still in remission and those who lost remission after discontinuing infliximab

There were no statistical significant differences in terms of the mean age, gender, disease duration, disease location, duration of disease prior to first infliximab infusion, age at first infliximab infusion, mean number of infliximab infusions, or mean duration of infliximab treatment between the CD patients who were still in remission after discontinuing infliximab (n = 6) and those who had lost remission after discontinuing their infliximab (n = 8) (Table 2).

The 8 patients who had lost remission after discontinuing infliximab had a mean number of 5 infusions (range 3-7), with a mean treatment time of 7.2 mo (range 1.5-15 mo). The mean duration of time from the last infusion of infliximab to the time of loss of remission was 382 d (range 20-701 d). The 6 patients who remained in remission after discontinuing infliximab had a mean number of 6 infusions (range 3-12), with a mean treatment duration of 12 mo (range 3.6-32 mo) (P = 0.45 relative to those who lost remission). Of the 8 patients who lost remission, 4 patients are currently on adalimumab, 2 patients are again on infliximab, 1 patient remains on azathioprine alone, and 1 patient is currently on no biological or immunomodulating agents due to a malignancy.

In contrast, there was a marked difference in the duration of clinical remission following discontinuance of infliximab between the two groups. The patients who lost remission did so after just 1.0 years ± 0.6 years, whereas those who were still in remission were at the time of this study, 8.1 years ± 2.6 years post-discontinuation of infliximab, $P < 0.001$ (Table 2). This marked difference confirms a group of patients that remained in remission for an extended interval of time after infliximab was discontinued while another group rapidly relapsed. We proceeded to genetically type these two groups.

Patient genotypes

Five SNPs of IBD5 and NOD2/CARD15 genes were successfully analyzed for all 14 subjects. There were no significant differences in SNPs between the group of patients who remained in remission for an extended interval of time after infliximab was discontinued, and the group of those who relapsed rapidly after infliximab was discontinued (Table 3).

DISCUSSION

Anti-TNF-α agents are well known to be effective for both induction and maintenance of remission in CD. However, no phenotypic features, environmental fac-

Table 3 5q31 and nucleotide-binding oligomerization domain 2/caspase activation recruitment domain 15 variants in crohn's disease patients still in remission and those who lost remission after discontinuing infliximab

Variant	Remaining in remission following discontinuing infliximab (n = 6)	Lost remission following discontinuing infliximab (n = 8)	Odds ratio	P value
Arg702Trp			1.50	1.00
Homozygous wild type, CC (%)	4 (67)	6 (75)		
Heterozygous, CT (%)	2 (33)	2 (25)		
Homozygous mutant, TT (%)	0	0		
Gly908Arg			-	-
Homozygous wild type, GG (%)	6 (100)	8 (100)		
Leu1007fs insC	2 (33)	2 (25)	1.50	1.00
IGR2060a1			1.67	1.00
Homozygous wild type, GG (%)	1 (17)	2 (25)		
Heterozygous, GC (%)	3 (50)	2 (25)		
Homozygous mutant, CC (%)	2 (33)	4 (50)		
IGR3081a1			-	0.47
Homozygous wild type, TT (%)	0	2 (25)		
Heterozygous, GT (%)	4 (66)	2 (25)		
Homozygous mutant, GG (%)	2 (33)	4 (50)		

tors, or clinical markers have been definitively shown to predict the duration of sustained clinical benefit after discontinuation of infliximab.

The lack of response to infliximab in patients with CD appears to be a stable occurrence over time, thus suggesting that infliximab response/non-response is related to a genetic predisposition[5]. Though over 100 genetic loci have been associated with IBD thus far, only two genes, *IBD5* and *CARD15*, have reliably been linked to CD susceptibility, and few genes have been proven to influence response to infliximab[12,19,21]. Although less than 20 percent of patients are homozygous for *CARD15* and the relative risk of developing CD for these individuals ranges from 10 to 40 percent, genetic markers remain important to understanding the complex pathogenesis of this disorder[22]. In comparison to biochemical markers such as C-reactive protein, genetic elements theoretically offer ideal markers for predicting disease outcomes, as genes are not affected by disease activity and are stable over time. As we more fully understand genetic variants and the roles they play in the development of CD, it is very plausible that certain genetic polymorphisms may be predictors of response to or relapse from anti-TNF-α agents such as infliximab.

Utilizing a well-defined cohort of patients with CD who were in an infliximab-induced corticosteroid-free clinical remission and then discontinued infliximab for reasons other than non-response[11], we studied two genetic variants of the IBD5 locus on chromosome 5q31 (IGR 2060a1 and IGR3081a1) along with three *NOD2/CARD15* polymorphisms (Arg702Trp, Gly908Arg, Leu1007fs insC), in those patients who had relapsed rapidly and compared them to those patients who remained in a long-term sustained remission.

Our study results show no significant increase in frequency of the *NOD2/CARD15* and IBD5 polymorphisms, for either the CD patients whose disease relapsed rapidly or for the CD patients who remained in a long-term sustained remission following the discontinuation of infliximab.

Urcelay *et al*[19] reported that the homozygous mutants in the 5q31 locus were related to a significant lack of response to infliximab and the frequency of these mutants was significantly higher in the non-responder group. Our study did not identify such findings, likely due to the fact that we were examining those infliximab-treated patients who had initially all responded to infliximab but after discontinuing infliximab fell into two groups: those that had a long-term sustained remission and those that rapidly relapsed.

Other studies have also shown no association between *NOD2/CARD15* genotype and response to infliximab[17,18]. In a large study of two CD patient cohorts with 534 participants (ACCENTI), Mascheretti *et al*[18] found no association between SNPs 8, 12 and 13 of the *CARD15* gene and infliximab response. SNP 8 includes the variant R702W and SNP12 includes G908R, which were also analyzed in our study. Genes encoding for TNF-α and TNF-α receptors have also been an area of some study, given the primary role of TNF-α in the inflammation of CD. A large cohort study by Louis *et al*[23] in 2002 did not show a CD response to infliximab amongst patients with the -308 polymorphism, a TNF-α promoter region mutation thought to influence TNF-α transcription. In another study focusing on the receptors TNFR1 and TNFR2, where TNF-α binds and exerts its effects, no clear effect of the polymorphisms on infliximab response was observed[24]. Again, the results from these previous investigations may not be comparable to those of the current study, in that we examined those infliximab-treated patients who initially responded to infliximab but who after discontinuing infliximab, fell into two groups: those that had a long-sustained response and those whose disease rapidly relapsed.

Of our 8 patients who experienced a flare of their disease after the discontinuation of infliximab, 4 are on adalimumab, 2 are on infliximab, 1 is only on azathioprine, and the remaining patient is on no therapy due

to active malignancy. Interestingly, of the 48 initially identified CD patients in the longitudinal cohort who were in an infliximab-induced clinical remission and then discontinued infliximab, approximately 16 patients (34%) remained in clinical remission a median of 477 d (95% CI; range of 290 to 1339 d; data from Waugh *et al*[11] as of July 2009. However, as time progressed, of these 16 patients who had remained in remission, 6 additional patients have now flared and are on alternative therapy as of July 2011 and only 10/48 (21%) remained in remission. This implies that even those patients who are in a long term sustained remission after discontinuing infliximab may ultimately relapse.

As we have seen with infliximab primary response, no genetic markers have been predictive of CD response to adalimumab. In a recent study assessing such a response to adalimumab, the same three NOD2 polymorphisms (Arg702Trp, Gly908Arg, Leu1007fs insC), in addition to TLR4 and CD14 mutations, were analyzed for 24 Spanish CD patients who had lost response to, or were unable to tolerate infliximab[25]. No association with any of these polymorphisms for the adalimumab response was noted.

There is a complex and not yet well-understood relationship between gene expression and disease behavior; indeed many genes remain to be identified in the case of CD. Of the genes identified so far, no variants have adequate sensitivity and specificity to be used in clinical decision making with biologic agent response[26]. Perhaps attention should be turned to pharmacogenetic studies, as despite being given optimal doses and duration of medications, 20 to 30 percent of IBD patients find their disease refractory to any given medication[26]. Currently, the only genetic test available in guiding clinical treatment decisions is that used for assessing risk of hematopoeitic toxicity from metabolism of thiopurine analogues by the enzyme thiopurine methyl transferase, which is under genetic control[27]. Polymorphisms of proteins targeted by drugs or enzymes involved in drug metabolism may be potential areas of useful study. However, limitations related to heterogeneity in IBD, such as disease severity, behaviour, and environmental factors affecting drug response, also make this a challenging area.

In the case of other autoimmune conditions such as rheumatoid arthritis, advancements have been made in predicting response to infliximab, specifically using whole-genome gene expression analysis with microarrays[27]. Julià *et al*[28] have presented an 8-gene model obtained from whole blood RNA samples with an 85.7% prediction accuracy of treatment response. However, like CD, no studies in rheumatoid arthritis have examined the genotypes responsible for relapse or remission after anti-TNF-α therapy has been stopped.

In conclusion, this study found that, in a cohort of infliximab-treated CD patients who had a sustained corticosteroid-free clinical remission and then discontinued infliximab, there are two groups; those who relapsed early usually within a year, and those who have a sustained clinical remission of up to a decade. Furthermore, this study was the first to attempt to identify genetic polymorphisms in these two subgroups, though the findings suggest no specific correlations for the polymorphisms tested.

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COMMENTS

Background

The etiology of Crohn's disease (CD) remains unclear and as with most complex-trait diseases, it involves a combination of genetic and environmental risk factors. Although over 100 genetic loci have been associated with inflammatory bowel disease (IBD) thus far, only two genes, *IBD5* and caspase activation recruitment domain 15 (*CARD15*), have reliably been linked to CD susceptibility. In terms of CD itself, a potent proinflammatory cytokine, tumor necrosis factor alpha (TNF-α), is a well-known mediator of inflammation. Furthermore, the appropriate duration of anti-TNF-α therapy for CD patients who are in remission is as yet undefined; specifically, the factors which affect the duration of sustained clinical benefit after discontinuation of infliximab remain poorly understood. This lends support to the possibility that a patient-unique genotype of CD may play a role in determining the duration of long-term remission after the discontinuation of anti-TNF-α agent therapy. Previous studies have analyzed genetic differences in infliximab responders and compared the results to infliximab non-responders, but the patient population does not include patients who have all initially responded to infliximab such as in this study.

Research frontiers

In the area of identifying genetic factors responsible for infliximab responses, the research hotspot has mainly focused on nucleotide-binding oligomerization domain 2 (*NOD2*)/*CARD15* genotypes and few studies have analyzed TNF-α receptor and promoter region polymorphisms. No genetic markers have been definitively predictive of CD response to infliximab.

Innovations and breakthroughs

A breakthrough in the area has been that over 100 genetic loci have been associated with IBD and thus far, two genes, *IBD5* and *CARD15*, have reliably been linked to CD susceptibility. However, few genes have been proven to influence response to infliximab. Utilizing a well-defined unique CD patient population who were in an infliximab-induced corticosteroid-free clinical remission and then discontinued infliximab for reasons other than non-response, the authors studied two genetic variants of the IBD5 locus on chromosome 5q31 (IGR 2060a1 and IGR3081a1) along with three *NOD2*/*CARD15* polymorphisms (Arg702Trp, Gly908Arg, Leu1007fs insC), in those patients who had relapsed rapidly and compared them to those patients who remained in a long-term sustained remission. These patients experienced either a sustained remission up to a decade or have relapsed rapidly often within a year. The study results show no significant increase in frequency of the *NOD2*/*CARD15* and *IBD5* polymorphisms for either group of CD patients. This study was the first to attempt to identify genetic differences in these two unique subgroups.

Applications

There is a complex and not yet well-understood relationship between gene expression and disease behavior. Anti-TNF-α agents such as infliximab are costly, and the long-term safety risks can be concerning in the long term. Furthermore, the appropriate duration of anti-TNF-α therapy for CD patients who are in remission is as yet undefined; specifically, no genes have been definitively identified to predict which CD patients who discontinued their infliximab while in full corticosteroid-free clinical remission might either have a sustained remission or will relapse rapidly after stopping infliximab.

Terminology

CARD15 was the first gene to be associated with CD; The *NOD2*/*CARD15* product is an intracellular protein involved in recognizing bacterial lipopolysaccharides in the nuclear factor kappa B-TNF-α pathway. This *NOD2*/*CARD15* gene is known to be associated with susceptibility to CD. IBD5 is a 250 kb haplotype in the 5q31 gene cluster associated with increased risk of CD.

Peer review

This is an interesting study comparing genetic differences between Crohn's disease patients who after discontinuing their infliximab while in full corticosteroid-free clinical remission, experienced a sustained remission versus those patients whose disease relapsed rapidly.

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BRIEF ARTICLE

Propionyl-L-carnitine hydrochloride for treatment of mild to moderate colonic inflammatory bowel diseases

Giuseppe Merra, Giovanni Gasbarrini, Lucrezia Laterza, Marco Pizzoferrato, Andrea Poscia, Franco Scaldaferri, Vincenzo Arena, Francesca Fiore, Achille Cittadini, Alessandro Sgambato, Francesco Franceschi, Antonio Gasbarrini

Giuseppe Merra, Francesco Franceschi, Emergency Department, Catholic University of Sacred Heart, 00168 Rome, Italy
Giovanni Gasbarrini, Medical Research Foundation and non-profit organization, 40123 Bologna, Italy
Lucrezia Laterza, Marco Pizzoferrato, Franco Scaldaferri, Antonio Gasbarrini, Department of Internal Medicine and Gastroenterology, Catholic University of Sacred Heart, 00168 Rome, Italy
Andrea Poscia, Institute of Hygiene, Catholic University of Sacred Heart, 00168 Rome, Italy
Vincenzo Arena, Institute of Pathology, Catholic University of Sacred Heart, 00168 Rome, Italy
Francesca Fiore, Department of Internal Medicine and Geriatry, Catholic University of Sacred Heart, 00168 Rome, Italy
Achille Cittadini, Alessandro Sgambato, Institute of General Pathology, Catholic University of Sacred Heart, 00168 Rome, Italy
Author contributions: Gasbarrini G and Gasbarrini A designed the research; Gasbarrini G evaluated the histological samples; Merra G, Fiore F and Franceschi F performed the research; Arena V provided technological and material support; Pizzoferrato M, Poscia A, Scaldaferri F, Cittadini A and Sgambato A analyzed the data; Merra G, Laterza L, Scaldaferri F and Sgambato A wrote and revised the paper.
Correspondence to: Antonio Gasbarrini, MD, Professor of Internal Medicine, Department of Internal Medicine and Gastroenterology, Catholic University of Sacred Heart, L.go Gemelli 8, 00168 Rome, Italy. agasbarrini@rm.unicatt.it
Telephone: +39-6-30156265 Fax: +39-6-30156265
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Abstract

AIM: To assess clinical and endoscopic response to propionyl-L-carnitine hydrochloride (PLC) in colonic inflammatory bowel disease.

METHODS: Patients suffering from mild to moderate ulcerative colitis (UC) or Crohn's disease (CD) colitis, with disease activity index (DAI) between 3 and 10 and under stable therapy with oral aminosalicylates, mercaptopurine or azathioprine, for at least 8 wk prior to baseline assessments, were considered suitable for enrollment. Fourteen patients were enrolled to assume PLC 2 g/d (two active tablets twice daily) orally. Clinical-endoscopic and histological activity were assessed by DAI and histological index (HI), respectively, following a colonoscopy performed immediately before and after 4 wk treatment. Clinical response was defined as a lowering of at least 3 points in DAI and clinical remission as a DAI score $\leqslant$ 2. Histological response was defined as an improvement of HI of at least 1 point. We used median values for the analysis. Differences pre- and post-treatment were analyzed by Wilcoxon signed rank test.

RESULTS: All patients enrolled completed the study. One patient, despite medical advice, took deflazacort 5 d before follow-up colonoscopy examination. No side effects were reported by patients during the trial. After treatment, 71% (SE 12%) of patients achieved clinical response, while 64% (SE 13%) obtained remission. Separating UC from CD patients, we observed a clinical response in 60% (SE 16%) and 100%, respectively. Furthermore 60% (SE 16%) of UC patients and 75% (SE 25%) of CD patients were in clinical remission after therapy. The median DAI was 7 [interquartile range (IQR): 4-8] before treatment and decreased to 2 (IQR: 1-3) ($P < 0.01$) after treatment. Only patients with UC showed a significant reduction of DAI, from a median 6.5 (IQR: 4-9) before treatment to 2 (IQR: 1-3) after treatment ($P < 0.01$). Conversely, in CD patients, although displaying a clear reduction of DAI from 7 (IQR: 5.5-7.5) before therapy to 1.5 (IQR: 0.5-2.5) after therapy, differences observed were not significant ($P = 0.06$). Seventy-nine percent (SE 11%) of patients showed improvement of HI of at least 1 point, while only one CD and two UC patients showed HI stability; none showed HI worsening. Median HI

decreased from 1 (IQR: 1-2), to 0.5 (IQR: 0-1) at the endoscopic control in the whole population ($P < 0.01$), while it changed from 1 (IQR: 1-2) to 0.5 (IQR: 0-1) in UC patients ($P < 0.01$) and from 1.5 (IQR: 1-2) to 0.5 (IQR: 0-1) in CD patients (P = not significant). The two sample tests of proportions showed no significant differences in clinical and histological response or in clinical remission between UC and CD patients. No side effects were reported during treatment or at 4 wk follow-up visit.

CONCLUSION: PLC improves endoscopic and histological activity of mild to moderate UC. Further studies are required to evaluate PLC efficacy in colonic CD patients.

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Key words: Propionyl-L-carnitine; Ulcerative colitis; Crohn's disease; Inflammatory bowel disease therapy; Propionyl-L-carnitine hydrochloride

Peer reviewers: Andrew S Day, Professor, University of Otago, Christchurch Hospital, Christchurch 8140, New Zealand; Dr. Ferenc Sipos, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi u. 46., 1088 Budapest, Hungary

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are idiopathic, immune-mediated and multifactorial diseases, including Crohn's disease (CD) and ulcerative colitis (UC)[1]. IBD pathogenesis has not been definitely clarified and UC and CD are considered complex diseases of unknown etiology, in which genetic and environmental factors force an abnormal intestinal mucosa immune reaction towards food and bacterial antigens[2-4]. Regardless of the main and unknown cause, several mechanisms sustain chronic inflammation and the vicious circle in IBD. Among those factors, energy deficiency[5-7] and peroxidative damage[8,9] of colonocytes play a pivotal role in IBD pathogenesis.

Propionyl-L-carnitine hydrochloride (PLC) is the (*R*)-3-(1oxo-propoxy)-4-(N,N,N-trimethyl amonium chloride) butanoic acid. It is a natural derivative of L-carnitine, produced in the body by the carnitine-acetyltransferase-mediated reaction between propionyl-CoA and L-carnitine. Physiologically, L-carnitine has an important role in energy regulation, taking part in fatty acid β-oxidation and glucose metabolism, and it has been used as a therapeutic option in conditions characterized by decreased ATP levels, such as heart failure and ischemia[10]. Moreover, PLC acts as an antioxidant molecule, reducing the lipid peroxidation in coronary endothelial cells[11-15]. Furthermore, PLC is a carrier of propionate moiety, which enters the Kreb's cycle, once transformed in succinate, contributing to energetic sources of the cell[16]. Finally, PLC has been used for the therapy of cutaneous trophic ulcers[17].

Based on these results, a similar effect of PLC in colonic IBD has been supposed; a condition characterized by peroxidative damage, energy impairment, ulcers and abnormal epithelial function of colonocytes. Beyond intriguing pathogenic considerations, the effect of PLC on IBD and in particular on UC patients, has been recently addressed by a positive phase II trial[18] on mild to moderate UC patients.

Carnitine transport deficiency could also play a role in CD pathogenesis, as suggested by genetic studies, showing mutations in genes coding for carnitine transporters, *OCTN1* (*SLC22A4*) and *OCTN2* (*SLC22A5*), in CD patients[19]. Furthermore, in a murine model with loss-of-function mutation in the *OCTN2* gene, the defect in carnitine transporter function in intestinal epithelial cells leads to atrophy of the small intestine and colon, and subsequent onset of inflammation[20].

Based on these considerations, in this proof-of-concept pilot study, we investigated the efficacy of PLC not only in UC, but also in colonic CD patients.

MATERIALS AND METHODS

This was a prospective, single center, open label, proof-of-concept pilot study on 14 patients, to investigate the efficacy of PLC (ST 261) colon release tablets at the dosage of 2 g/d in patients affected by mild to moderate colonic IBD, with stable oral treatment.

The protocol was approved by the Ethics Committee of "A. Gemelli" Hospital, Rome, Italy. The trial was conducted according to the Helsinki Declaration and the Good Clinical Practice Guidelines adopted by the European Medicines Agency in 1996. Every patient signed a written informed consent.

Study endpoints

Primary endpoint of the study was to evaluate the percentage of patients displaying clinical/endoscopic response following 4 wk of stable therapy, defined as a lowering of the disease activity index (DAI score, Table 1) of at least 3 points upon the baseline. Percentage of patients reaching clinical remission, defined by a DAI score $\leqslant 2$ with no individual subscore > 1, was also evaluated. As secondary endpoint, histological response to the treatment was assessed, defined as an improvement of the histological index (HI) of at least 1 point at the end of the study compared to baseline.

Inclusion and exclusion criteria

Patients suitable for the enrollment met all the following

Table 1 Disease activity index evaluation according to Mayo score

	Stool frequency	Mucosal appearance	Rectal bleeding	Physician's overall assessment of disease severity
0	Normal number of stools for the patient	Normal	None	Normal
1	1-2 stools/d more than normal	Mild friability	Streaks of blood	Mild
2	3-4 stools/d more than normal	Moderate friability	Obvious blood	Moderate
3	5 or more stools/d more than normal	Exudation, spontaneous bleeding	Mostly blood	Severe

Score subtotal on the left column.

inclusion criteria: age between 16 and 80 years; if female, not pregnant or nursing; for women of childbearing potential, willingness to avoid a pregnancy during the treatment period and for at least 1 mo from the last dose of drug; availability of pancolonoscopy and histology both confirming the diagnosis of active inflammatory bowel disease; DAI between 3 and 10 (mild to moderate disease); under stable therapy with oral aminosalicylates (mesalazine, balsalazide, olsalazine or sulfasalazine for ⩾ 8 wk prior to baseline assessments), mercaptopurine or azathioprine for ⩾ 8 wk prior to baseline assessments.

The exclusion criteria were: first diagnosis of IBD; known CD of the small intestine or of other sites rather than the colon; current or previous (in the past 30 d before the screening) use of systemic corticosteroids; use of antibiotics in the past 7 d before the screening; use of NSAIDs in the past 7 d preceding screening; positive stool culture (when performed, according to investigator's judgment, to assess possible parasitological infection); significantly impaired liver, renal, pulmonary or cardiovascular function; history of total colon resection; active or chronic infection.

Study design

During a screening phase of 2 wk, each patient was clinically evaluated and underwent colonoscopy with biopsy sampling for histology, to assess HI and DAI score. Clinical/endoscopic evaluation was performed using DAI, a validated index[18] for UC patients, whose criteria are reported on Table 1. In this trial, we also used DAI to assess Crohn's colitis. In agreement with its use in other clinical trials, a DAI score of 3-10 was considered evidence of a mild (3-6) to moderate (7-10) disease[18].

After the enrollment, patients were administered with two PLC colon release 500 mg tablets twice daily (1 h before breakfast and 1 h before dinner) for a total daily dose of 2 g, for 4 wk. After 4 wk of treatment, at a control visit, each patient underwent colonoscopy with biopsy sampling for histology and clinical evaluation with assessment of HI and DAI score. After 4 wk since the last visit, a follow-up visit was performed to assess safety of the treatment.

To assess safety and tolerability, vital signs, physical examinations, electrocardiograms and laboratory tests were registered at each visit (at the enrollment and after 4 and 8 wk).

Histological score

All histological scores were calculated by a single expert pathologist, following histological criteria described in Mikhailova *et al*[18] and modified from Truelove *et al*[21]. Briefly, inflammation was classified as grade 0: if indistinguishable from normal intestinal mucosa or, where structural features of UC were present, there were no polymorphs in the crypt epithelium; grade 1: if there was an increased number of leukocytes in the lamina propria and initial infiltration of crypt epithelium, but no abscesses or epithelial erosions were present; grade 2: if crypt abscesses (mandatory) and erosive foci, but no ulcerative lesions, were detected; and grade 3: if ulcerative lesions were clearly evident. The same score was also used to classify colonic CD. Lowering of HI by at least 1 point was considered as histological response.

Statistical analysis

Statistical analysis was performed by using IC STATA Release 12.0 for MAC. Data were reported as absolute numbers, percentage, median and interquartile ranges (IQRs), as specified. Mann-Whitney test was used to compare UC and CD patients. Differences were analyzed by Wilcoxon signed rank test, while the proportion of binary variables (clinical or histological response/clinical remission) between UC and CD patients was evaluated by a two-sample test of proportions. $P < 0.05$ was considered statistically significant. We used median values for the analysis, because of the small number of patients enrolled, and we preferred nonparametric tests.

RESULTS

Fourteen patients were enrolled. The mean age, female/male ratio and demographics are indicated in Table 2. With regard to therapy, six patients were treated with mesalamine 2.4 g/d; three with azathioprine at 2 mg/kg; and one with 7.5 mg/d deflazacort, starting 5 d before follow-up colonoscopy, despite medical advice to the contrary; the remaining four patients did not receive any treatment. CD patients were overall younger than UC patients and they were all male, but they were comparable to UC patients in term of DAI and HI at the time of enrollment. However, no significant differences were detected between UC and CD patients in term of demographic data.

Efficacy of PLC on clinical/endoscopic disease activity

After treatment, 71% (SE 12%) and 64% (SE 13%) of patients achieved a clinical response, or obtained remission, respectively. Separating UC patients from CD patients, we observed a clinical response after therapy

Table 2 Features of patients enrolled into the study

Patients features	All	UC	CD
No. of patients	14	10	4
Sex (F/M)	5/9	5/5	0/4
Age (yr), mean ± SD	49.4 ± 17.7	54.9 ± 17.5	34.7 ± 7.6
Disease duration at enrollment (yr), mean ± SD	4.8 ± 3.1	4.9 ± 3.1	4.5 ± 3.7
Diagnosis			
Pancolitis	8	-	-
Distal colitis	2	-	-
Colonic CD	4	-	-
DAI at time of enrollment, median ± SE	7 ± 4	6.5 ± 5	7 ± 2
DAI after the treatment, median ± SE	2 ± 2	2 ± 2	1.5 ± 2
HI at time of enrollment, median ± SE	1 ± 1	1 ± 1	1.5 ± 1
HI after the treatment, median ± SE	0.5 ± 1	0.5 ± 1	0.5 ± 1
DAI at time of enrollment, mean ± SD	6.6 ± 2.4	6.6 ± 2.7	6.5 ± 1.7
DAI after treatment, mean ± SD	1.9 ± 1.5	2.0 ± 1.6	1.5 ± 1.3
HI at time of enrollment, mean ± SD	1.4 ± 0.5	1.4 ± 0.5	1.5 ± 0.6
HI after treatment, mean ± SD	0.5 ± 0.5	0.5 ± 0.5	0.5 ± 0.6
Time between 1st and 2nd colonoscopy (wk), mean ± SD	4.7 ± 4	5.1 ± 4.6	3 ± 2.8
Other therapy (*n*)			
Mesalazine	6	5	1
AZA	3	1	2
Deflazacort	1	1	0
No other therapy	4	3	1

AZA: Azathioprine; F: Female; M: Male; UC: Ulcerative colitis; CD: Crohn's disease; DAI: Disease activity index; HI: Histological index.

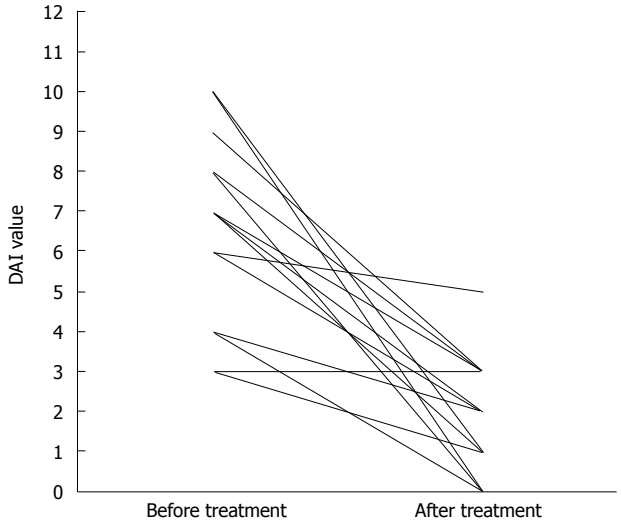


Figure 1 Disease activity index before and after treatment in all patients. In the left part of the graph, there are disease activity index (DAI) values for every patient affected by ulcerative colitis or Crohn's disease at enrollment. In the right part of the graph, there are DAI values for every patient after treatment.

in 60% (SE 16%) of UC patients and in 100% of CD patients; furthermore 60% (SE 16%) of UC patients and 75% (SE 25%) of CD patients were in clinical remission after therapy. The median DAI was 7 (IQR: 4-8) before treatment and decreased to 2 (IQR: 1-3) ($P < 0.01$) after treatment (Table 2 for mean values and Figure 1 for details). Stratifying by disease, only patients with UC showed a significant reduction of DAI, from a median

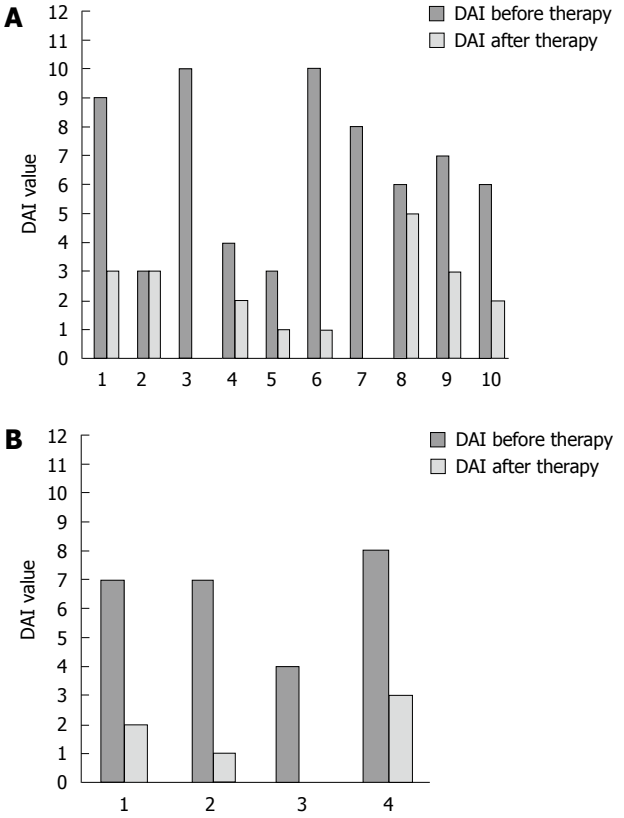


Figure 2 Disease activity index in patients affected by ulcerative colitis and Crohn's disease. A: The dark grey columns show disease activity index (DAI) values before treatment, while the light grey columns show DAI values after treatment in patients with ulcerative colitis; B: The dark grey columns show DAI values before treatment, while the light grey columns show DAI values after treatment in patients with Crohn's disease.

value of 6.5 (IQR: 4-9) before treatment to a median value of 2 (IQR: 1-3) ($P < 0.01$) after treatment (Figure 2A). Conversely, in CD patients, although displaying a clear reduction of DAI from 7 (IQR: 5.57-5) before therapy to 1.5 (IQR: 0.5-2.5) after therapy, differences observed were not significant ($P = 0.06$) (Figure 2B).

Efficacy of PLC on ameliorating HI

Overall, 79% (SE 11%) of patients showed an improvement in HI of at least 1 point, while only one CD and two UC patients showed HI stability; none showed HI worsening (Figure 3). Median HI decreased from 1 (IQR: 1-2), to 0.5 (IQR: 0-1) at the endoscopic control in the whole population ($P < 0.01$), while it changed from 1 (IQR: 1-2) to 0.5 (IQR: 0-1) in UC patients ($P < 0.01$) (Figure 4A) and from 1.5 (IQR: 1-2) to 0.5 (IQR: 0-1) in CD patients (P = not significant) (Figure 4B). The two-sample tests of proportions showed no significant differences in clinical/histological responses or clinical remissions between UC and CD patients.

Safety

No side effects were reported during treatment or at 4 wk follow-up visit.

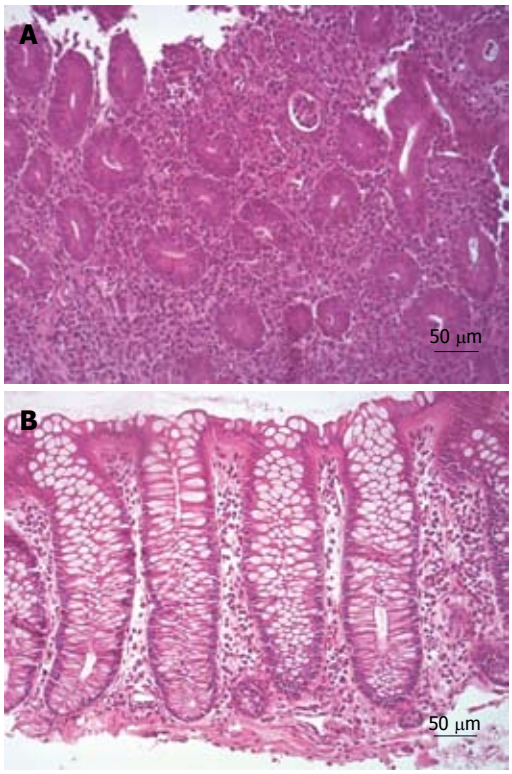


Figure 3 Histological samples before and after therapy. A: Biopsy specimen showing crypt abscess and perycriptitis (histological grade 2); B: Biopsy specimen morphologically indistinguishable from normal intestinal mucosa (histological grade 0).

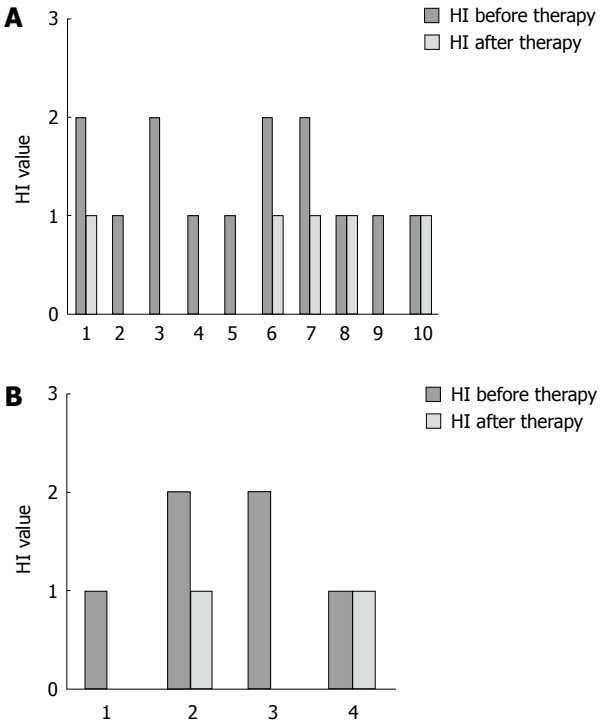


Figure 4 Histological index in patients affected by ulcerative colitis and Crohn's disease. A: The dark grey columns show histological index (HI) values before treatment, while the light grey columns show HI values after treatment in patients with ulcerative colitis; B: The dark grey columns show HI values before treatment, while the light grey columns show HI values after treatment in patients with Crohn's disease.

DISCUSSION

We enrolled 14 patients who were under stable oral therapy, without a complete control of symptoms and with evidence of endoscopic/histological disease. Therapeutic options sustained by strong clinical data for these patients are nowadays particularly limited.

Each patient underwent colonoscopy with biopsy sampling for histology and clinical evaluation with calculation of DAI score before starting treatment and repeated the same evaluation after 4 wk of therapy with PLC (ST 261) colon release tablets at a dose of 2 g/d. Following active treatment, the majority of them displayed a clear clinical improvement (70% of patients achieved clinical response) and 79% of patients presented with histological improvement. Our study confirmed the good clinical efficacy of PLC, in contrast to other studies using only SCFA or butyrate enemas in patients with more severe forms of UC[22-25]. Our results confirmed preclinical data showing the efficacy of PLC in reducing the damaged colon area in acute and chronic models of murine colitis. In humans, PLC given intrarectally shows good results in improving clinical and histological activity in patients suffering from UC[26]. Oral formulation of PLC is more agreeable for patients, and probably improves patient compliance with therapy.

Our single center study confirms the results from a multicentre phase Ⅱ, double-blind, parallel-group trial by Mikhailova *et al*[18] in patients suffering from UC. That trial showed a clinical/endoscopic response rate of 75% in patients exposed to PLC 1 g/d and a response rate of 69% in patients exposed to PLC 2 g/d, with remission rates of 55%, 49% and 35% respectively for PLC 1 g, 2 g and placebo groups. Histological responses rates were 40%, 33% and 35%, respectively. Histological assessment, however, was not significantly different among the groups. We showed a similar clinical/endoscopic remission rate and, moreover, a higher histological response rate after therapy. Furthermore, we also investigated the efficacy of PLC in CD patients, who showed a significant clinical/endoscopic response.

Nevertheless, our study had several limitations. This was an open label study, without a placebo group. Furthermore, one patient, considered in the final analysis, took 7.5 mg/d deflazacort for 5 d, despite medical advice, just before the follow-up endoscopy evaluation. This particular patient had UC, but was still considered for the final analysis because we did not consider this duration and dose of deflazacort to affect clinical and histological outcome significantly, for the purpose of our study. Furthermore, our patients, as happens frequently in clinical practice, were receiving concomitant therapy with mesalamine and/or azathioprine. The limited number of patients did not permit a stratification based on use of concomitant medication. However, we can exclude that the clinical response and remission in these patients were dependent on other therapy, which

remained unchanged for at least 2 mo before enrollment and throughout the study. Patients enrolled in this study were a mixed population of UC and colonic CD patients. One can argue that these different diseases would lead to nonhomogenous results among subgroups and potentially different responses, but the number of patients enrolled was too small to allow subgroup analysis. Therefore, with our data, we cannot make conclusions about different responses to treatment in UC and colonic CD.

However, based on the supposed mechanisms of action of this drug, we expected a response in both UC and CD. In fact colonocytes from UC and colonic CD patients, showing defect in carnitine functions, would probably both benefit from PLC therapy.

Compared with the study of Sandborn *et al*[27], the present trial suggests a possible role of this drug in colonic CD patients, even if our results in these patients did not reach statistical significance; probably because of the small number of patients. Nevertheless, the clinical response in CD patients was good and, despite the limitation of the absence of a placebo arm, it can be argued that it was higher than expected from the response to a placebo arm, as based on the literature. For this reason, this proof-of-concept pilot study lays the foundations for future studies on the use of PLC in colonic CD patients.

In conclusion, PLC (ST 261) colon release tablets at a dose of 2 g/d were effective in inducing clinical and endoscopic/histological improvement in mild to moderate colonic IBD. Other studies are required to define the real efficacy and the correct therapeutic dosage of this promising drug for both colonic forms of IBD, and specially to evaluate its efficacy in CD patients.

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In memory of Dr. Claudio Cavazza, for his love for research and for having believed in this project.

COMMENTS

Background

There are few options in inflammatory bowel disease (IBD) therapy, mainly because pathogenetic mechanisms of these diseases are still unclear. Several components could play a role in IBD pathogenesis, including peroxidative damage, energy impairment and abnormal epithelial function on colonocytes. Propionyl-L-carnitine hydrochloride (PLC) modulation of these impaired functions could be useful in IBD therapy.

Research frontiers

PLC has demonstrated efficacy in reducing clinical and endoscopic disease activity in patients suffering from ulcerative colitis (UC), but only a few studies have investigated its use. Based on its mechanism of action, this drug could be effective also in patients affected by Crohn's disease (CD), but no clinical trials have assessed the efficacy of this drug in CD.

Innovations and breakthroughs

This is one of the first studies to investigate the use of PLC in patients suffering from UC and CD. The data suggest that this drug demonstrates efficacy and safety in both conditions.

Applications

With regard to UC, these data are in accordance with previous findings emerging from a multicenter, double-blind, placebo-controlled clinical trial. Furthermore, they suggest a possible use of this drug also in CD colitis and, even if few patients have been treated, this proof-of-concept study lays the foundations for future wider clinical trials.

Terminology

PLC has an important role in energy regulation, taking part in fatty acid β-oxidation and glucose metabolism. Moreover, PLC acts as an antioxidant molecule and it is a carrier of propionate moiety, which enters the Kreb's cycle, once transformed in succinate, contributing to energy sources in the cell. Finally, PLC has shown efficacy in treatment of cutaneous trophic ulcers.

Peer review

This is an interesting and important study that provides clinical evidence of the benefit of this novel therapy in patients with colitis. The topic is hot and of great clinical importance and these promising data would support further clinical assessment of this agent.

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BRIEF ARTICLE

Prognostic value of 18-fluorodeoxyglucose positron emission tomography-computed tomography in resectable colorectal cancer

Jang Eun Lee, Sang Woo Kim, Jin Su Kim, Kyu Yong Choi, Won Kyung Kang, Seong Taek Oh, Ie Ryung Yoo, Sung Hoon Kim

Jang Eun Lee, Sang Woo Kim, Jin Su Kim, Kyu Yong Choi, Department of Internal Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of South Korea, Seoul 137-701, South Korea
Won Kyung Kang, Seong Taek Oh, Department of Surgery, College of Medicine, The Catholic University of South Korea, Seoul 137-701, South Korea
Ie Ryung Yoo, Sung Hoon Kim, Department of Radiology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of South Korea, Seoul 137-701, South Korea
Author contributions: Lee JE and Kim SW designed the study and analyzed the data; Lee JE, Kim SW, Kim JS and Choi KY analyzed the date and contributed to the interpretation; Kang WK and Oh ST performed surgery and contributed to the materials; Yoo IR and Kim SH reviewed the FDG-PET/CT and contributed to the materials; and Lee JE and Kim SW wrote the manuscript.
Correspondence to: Sang Woo Kim, MD, Department of Internal Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of South Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, South Korea. viper@catholic.ac.kr
Telephone: +82-2-22582083 Fax: +82-2-22582089
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Abstract

AIM: To assess the prognostic value of preoperative 18 fluorodeoxyglucose positron emission tomography (FDG-PET)/computed tomography (CT) in patients with resectable colorectal cancer.

METHODS: One hundred sixty-three patients with resectable colorectal cancer who underwent FDG-PET/CT before surgery were included. Patient data including pathologic stage at presentation, histology, treatment, disease-free survival and the maximum standardized uptake value (SUVmax) of the primary tumor on FDG-PET/CT were retrospectively analyzed. Median follow up duration was 756 (range, 419-1355). The primary end point was disease-free survival.

RESULTS: Twenty-five of 163 patients (15.3%) had recurrences. The median SUVmax values of the recurrence and no-recurrence groups were 8.9 (range, 5-24) and 8.2 (range, 0-23, P = 0.998). Receiver operating characteristic (ROC) curve analysis showed no significant association between SUVmax and recurrence (area under the curve = 0.5, P = 0.998, 95% CI: 0.389-0.611). Because a statistically significant value was not found, SUVmax was dichotomized at its median of 8.6. The disease-free survival curve was analyzed using the median SUVmax (8.6) as the cut off. Univariate and multivariate analysis did not provide evidence that disease-free survival rates for the subgroups defined by the median SUVmax were significantly different (P = 0.52, P = 0.25).

CONCLUSION: Our study suggests that the high FDG uptake of primary mass in resectable colorectal cancer doesn't have a significant relationship with tumor recurrence and disease-free survival.

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Key words: Positron-emission tomography; Colorectal neoplasms; Disease-free survival; Recurrence; Prognosis

Peer reviewers: Shu Zheng, Professor, Cancer Institute, Zhejiang University, No.88 Jiefang road, Hangzhou 310009, Zhejiang Province, China; Dr. Freddy Penninckx, Professor, Department of Abdominal Surgery, University Clinic Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium

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INTRODUCTION

Colorectal cancer is the third most common malignancy and the second most frequent cause of cancer-related deaths in the United States, with approximately 51 370 cancer-related deaths in 2010[1]. Despite improvement in diagnosis and treatment modalities, mortality from colon cancer has decreased slightly over the past 30 years. Therefore, methods for identifying patients at high risk of recurrence could influence posttreatment surveillance with the ultimate goal of improving survival.

Fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET) is a functional imaging method for measurement of tumor glucose use. FDG-PET combined with computed tomography (FDG-PET/CT) was recently introduced and is expected to provide more precise anatomical data along with metabolic information. FDG-PET/CT imaging may be useful for diagnosis of patients with malignant disease, assessment of the extension of disease, detection of tumor recurrence and for monitoring responses to therapy[2-5].

In addition, some researchers have suggested that metabolic activity on FDG-PET may be a predictive marker for some types of malignancies[6-9]. Recent studies have demonstrated that there is a correlation between the maximum standardized uptake value (SUVmax) and survival in patients with gastrointestinal cancers such as esophageal and stomach cancer[10-12]. However, the prognostic value of FDG-PET/CT for colorectal cancer is less well studied. Several studies have found that metabolic activity on FDG-PET/CT is correlated with tumor biology and survival in colon cancer[13-15]. However, these studies were limited by experimental studies, small numbers of patients, and one report focused on stage Ⅳ colorectal cancer[14]. Therefore, in this study, we evaluated FDG-PET/CT imaging of patients with resectable colorectal cancer and determined whether metabolic activity is correlated with recurrence and disease-free survival.

MATERIALS AND METHODS

Patients

We retrospectively reviewed the tumor registry at our institution and identified all patients who were diagnosed with colorectal cancer and underwent curative resection between January 2006 and June 2008. Patients were required to have undergone the FDG-PET/CT study before surgery and to have received no treatment before the FDG-PET/CT. Primary colorectal cancer was confirmed by endoscopic biopsy. Patients who received neoadjuvant treatment or had a previous history of another malignancy were excluded. Patients with colorectal perforation or less than 1 year of follow-up were also excluded. Patients who did not receive adjuvant therapy despite lymph node-positive cancer were excluded from the analysis.

All patients underwent full imaging of the colon by colonoscopy unless an obstructing lesion was encountered. In this instance, colonoscopy was performed after recovery from surgical treatment. All surgeries were performed by two experienced colorectal surgeons.

Data were collected on demographic details, tumor node metastasis (TNM) and Duke staging at presentation, recurrence, histology, survival, disease-free survival, and SUVmax of the primary tumor. Tumor stage was classified according to the sixth edition of the TNM classification of the Union for International Cancer Control[16].

A total of 163 patients (65 women and 98 men) were eligible for this study. The median age of the patients was 60 years (range, 30 to 81 years).

Patient anonymity was preserved and the Institutional Review Board of Seoul St. Mary's Hospital approved the study. The study protocol was in complete compliance with the Declaration of Helsinki, as revised in Edinburgh in 2000.

Positron emission tomography imaging

All patients were fasted for at least 6 h before FDG injection; their blood glucose levels were determined from capillary blood samples collected before intravenous FDG injection. In our institution, the cut-off blood glucose level that contraindicates FDG injection is 8 mmol/L.

PET images were acquired 1 h after injection of 370-570 MBq of 18F-FDG. The patients were scanned generally from the base of the skull to the upper thighs with their arms raised above their heads.

FDG/PET-CT images were reviewed using fusion software (Syngo, Siemens; Knoxville, TN). An experienced nuclear medicine physician reviewed the PET/CT images. PET, CT and fused whole-body images displayed in axial, coronal, and sagittal planes were available for review. The PET data were also displayed as a rotating maximum-intensity projection. Abnormal FDG uptake, SUVmax of the primary tumor and distant metastases were evaluated.

Treatment and follow-up

All patients who were included in this study underwent curative resection based on a standardized technique and principle, which were not changed during the period of this study. Curative resection was defined as achieving complete microscopic/macroscopic clearance with no evidence of metastatic spread (except resectable liver or lung metastasis) at the time of surgical resection. The surgical margins were reported by experienced pathologists and all the margins were clear.

After curative resection of the tumor, adjuvant chemotherapy or chemoradiotherapy were indicated for all patients with lymph node-positive colorectal cancer. Patients were scheduled for follow-up visits every 3-6 mo for the first 2 years, every 6-12 mo for up to 4 years, and

Table 1 Baseline characteristics of the 163 patients

Characteristic	Value
Age (yr), median (range)	60 (30-81)
Male/female, *n*	98/65
Stage, *n*	
Ⅰ	23
Ⅱ	67
Ⅲ	64
Ⅳ	9
Follow-up duration (d), median (range)	756 (419-1355)
Recurrence, *n*	25
Death, *n*	3

annually thereafter. Follow-up evaluations included physical examinations at each visit, analysis of carcinoembryonic antigen (CEA) levels, abdominal and pelvic CT scans, and chest X-rays. A colonoscopy was performed 1 year after resection and then as indicated clinically. Abnormal physical findings or laboratory results mandated further screening with ultrasonography, CT, magnetic resonance imaging, or FDG-PET/CT, as indicated according to the clinician's decision.

Sample size calculation

In the previous study, the 2-year survival rates for an SUVmax of less than 10 and an SUVmax of 10 or more were 90% and 65%, respectively[14]. Based on the findings of our previous report, we hypothesized that the high SUVmax group would have a higher recurrence rate (the assumed odds ratio was 3 or more). The assumed odds ratio was decided based on the previous study for other malignancies, such as lung cancer and stomach cancer. We calculated that 163 patients would be required to detect these differences with an alpha level of 0.05 and a power of 0.80.

Statistical analysis

The predictive value of SUVmax for tumor recurrence was determined by analysis of the area under the receiver operating characteristic (ROC) curve. Because a statistically significant value was not found, SUVmax was dichotomized at its median of 8.6. The significance of the predictive value was analyzed by log-rank testing in univariate analysis and by Cox proportional hazards regression testing in multivariate analysis.

For statistical analysis, group means were compared using the Mann-Whitney or χ^2 tests, and disease-free survival was evaluated using a Kaplan-Meier survival analysis. For all tests, a P value of < 0.05 was considered statistically significant. All statistical analysis were performed using the Statistical Package for Social Sciences (SPSS), version 16 (SPSS, Chicago, IL).

RESULTS

Patient characteristics

The characteristics of the 163 patients enrolled in this study are shown in Table 1. The pathologic tumor stages were stage I in 23 patients, stage Ⅱ in 67 patients, stage Ⅲ in 64 patients, and stage Ⅳ in nine patients. Nine patients with stage Ⅳ tumors had metastases confined to the liver. Metastatic nodules on the liver numbered 1 or 2. The median size of the metastatic nodules was 1.2 cm (range, 0.5-2.5 cm). During the follow-up, recurrence was observed in 25 of 163 patients (15.3%). Three patients died with a median follow-up of 25.2 mo.

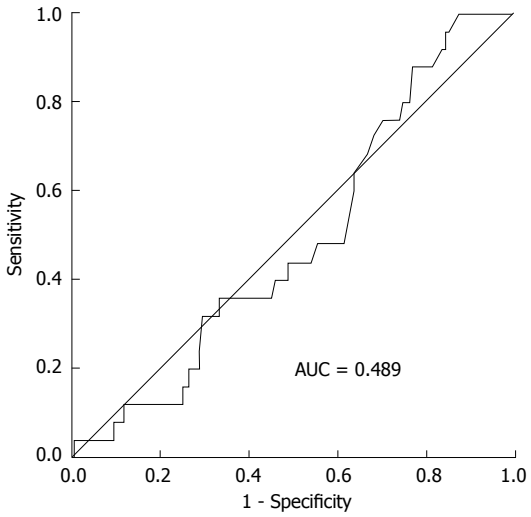


Figure 1 Receiver operating characteristic curve for prediction of recurrence from the maximum standardized uptake value. AUC: Area under curve.

SUVmax and recurrence

The median of SUVmax of the primary tumor was 8.6. ROC curve analysis showed no significant association between the SUVmax and recurrence (area under the curve = 0.5, P = 0.998, 95% CI: 0.389-0.611; Figure 1). As survival in patients with colorectal cancer is stage related, ROC curve analysis was conducted according to tumor stage. Patients with stage I tumors were excluded because they had no recurrence. Three ROC curves (stage Ⅱ and Ⅲ). None showed a statistically significant result. ROC curves according to stage showed that SUVmax was not a valid marker for predicting recurrence. Because a statistically significant value was not found, SUVmax was dichotomized at its median of 8.6. The disease-free survival curve was analyzed using the median SUVmax (8.6) as the cut off (Figure 2).

In a comparison between two groups differing in median SUVmax, tumor size (P = 0.02) and T stage (P = 0.018) showed significant differences. However, tumor recurrence, N stage, M stage, and histologic grade showed no significant differences (Table 2).

A disease-free survival curve calculated according to the Kaplan-Meier method for SUVmax of the primary tumor is shown in Figure 3 and Table 3. The difference in disease-free survival of patients categorized by median SUVmax was not statistically significant (P = 0.53).

After multivariate survival analysis, N stage and elevated CEA were independent prognostic predictors for disease-free survival (Table 4).

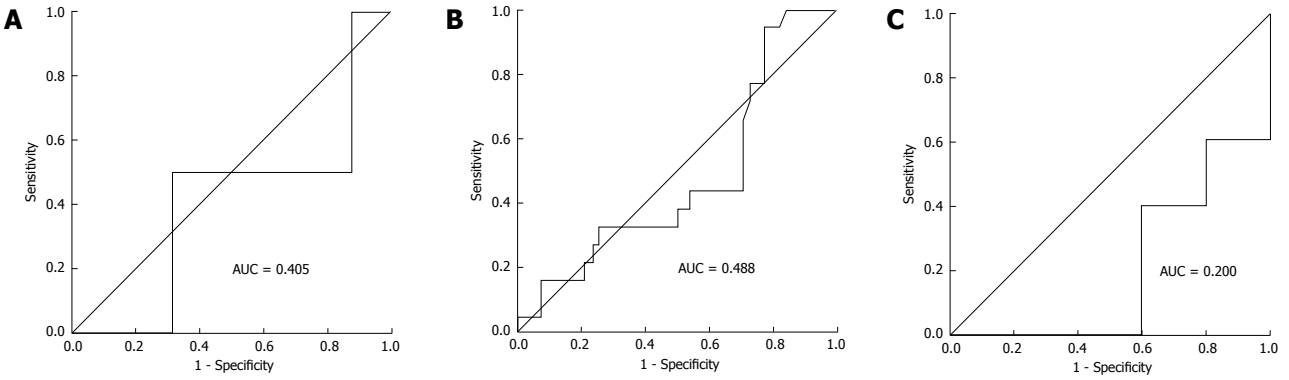


Figure 2 Receiver operating characteristic curves for prediction of recurrence from the maximum standardized uptake value and subgroup analysis according to stage. A: Subgroup analysis according to stage Ⅱ; B: Subgroup analysis according to stage Ⅲ; C: Subgroup analysis according to stage Ⅳ. AUC: Area under curve.

Table 2 The maximum standardized uptake value and clinicopathologic characteristics

	SUVmax < 8.6 (*n* = 80)	SUVmax ⩾ 8.6 (*n* = 83)	*P* value
Age (yr), median (range)	61.0 (37-81)	60.0 (30-81)	0.799
Sex (male), *n*	47	51	0.872
Stage, *n*			0.346
Ⅰ	15	8	
Ⅱ	33	34	
Ⅲ	28	36	
Ⅳ	4	5	
Tumor size (cm), median (range)	4.0 (2-13.5)	4.5 (2.4-9.5)	0.02
T stage, *n*			0.01
1	5	3	
2	12	8	
3	59	57	
4	4	15	
N stage, *n*			0.955
0	47	44	
1	17	24	
2	16	15	
M stage, *n*			0.562
0	76	78	
1	4	5	
Histology			0.54
WD	6	7	
MD	71	67	
PD		4	
Mucinous	3	5	
Median follow-up (d)	776	756	0.285
Recurrence, *n*	14	11	0.509
Death, *n*	2	1	0.547

SUVmax: Maximum standardized uptake value; WD: Well-differentiated adenocarcinoma; MD: Moderately differentiated adenocarcinoma; PD: Poorly differentiated adenocarcinoma; T: Tumor; N: Node; M: Metastasis.

Table 3 Two-year disease-free survival according to the Kaplan-Meier method

Variable	No. of patients	Two-year DFS (%)	Log-rank *P*
Age (yr)			0.29
⩾ 60	81	90.1	
< 60	82	85.4	
Sex			0.08
Male	98	83.7	
Female	65	93.8	
TNM stage			< 0.01
Ⅰ	23	100	
Ⅱ	67	98.5	
Ⅲ	64	78.1	
Ⅳ	9	44.4	
SUVmax			0.53
⩾ 8.6	83	86.7	
< 8.6	80	88.8	
CEA (ng/mL)			< 0.01
> 5	36	72.2	
⩽ 5	123	93.5	

DFS: Disease-free survival; TNM: Tumor node metastasis; SUVmax: Maximum standardized uptake value; CEA: Carcinoembryonic antigen.

Table 4 Multivariate Cox regression analysis of disease-free survival

Variable	Relative risk	95% CI	*P* value
SUVmax ⩾ 8.6	0.8	0.35-2.16	0.76
T stage (T3, T4)	3.1	0-6.6	0.94
N stage (N2)	9.78	2.01-49.5	0.01
M stage (M1)	2.51	0.78-7.98	0.12
Age ⩾ 60 yr	0.6	0.35-2.16	0.35
Male	1.9	0.71-5.15	0.20
CEA > 5 ng/mL	3.2	1.26-8.42	0.02

SUVmax: Maximum standardized uptake value; CEA: Carcinoembryonic antigen; T: Tumor; N: Node; M: Metastasis.

DISCUSSION

FDG-PET/CT has been proposed for use in the diagnosis and staging of several malignancies[4]. Furthermore, recent studies have demonstrated that there is a correlation between SUVmax and survival in patients with gastrointestinal cancers such as esophageal and stomach cancer[10-12].

This study focused on the role of FDG-PET/CT in providing prognostic information on patients with resectable colorectal cancer. We analyzed whether the SUVmax in the primary mass was associated with recurrence and disease-free survival. Our study demonstrates that the SUVmax of the primary mass in patients with resectable colorectal cancer provides no prognostic information.

FDG-PET/CT is a molecular imaging tool that monitors tissue glucose metabolism[17]. The biologic correla-

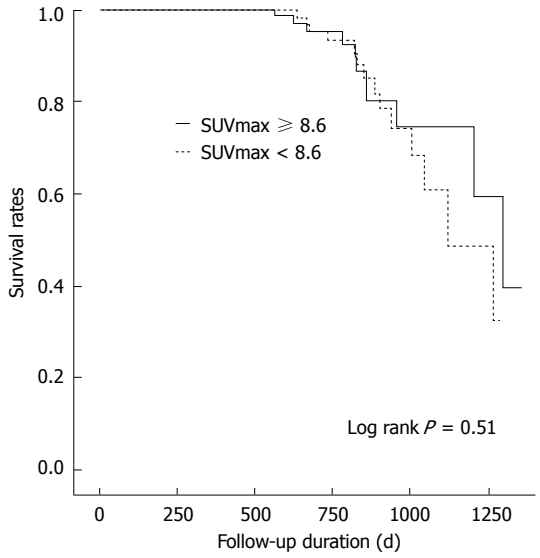


Figure 3 Disease-free survival according to the maximum standardized uptake value. SUVmax: Maximum standardized uptake value.

tions of FDG accumulation are less well studied for colorectal cancer than for other cancers. That cellular glucose metabolism may be correlated with tumor growth and aggressiveness has been suggested in some studies[17-21]. Previous *in vitro* and clinical studies indicated an association between elevated FDG uptake and colon tumor glucose metabolism, such as the activity of GLUT1, Ki-67, and p53[13,22-25]. A study by Gu *et al*[22] indicated that tumor size and depth of invasion were associated with higher SUVs in both primary colon tumors and hepatic metastatic foci. However, the study was limited by a small sample size (20 patients) and survival analysis was not performed. The other report demonstrated that a high SUVmax is correlated with poor prognosis and predicts mortality for patients with colorectal cancer after liver metastasectomy[14]. However, in this study, the highest SUVmax value was selected regardless of whether the lesion was primary or metastatic. In addition, the authors did not state whether complete resection was achieved and multivariate analysis of prognostic factors was not performed. Past studies that showed a correlation between survival and SUV were focused on stage Ⅳ colorectal cancer. In this study, we included only resectable colorectal cancers, in which clinicians are most interested. The main prognostic factors for disease-free survival were tumor stage, especially nodal stage, and elevated CEA. The SUVmax of the primary tumor was not a significant prognostic factor.

There are several limitations to this study. First, this study was a retrospective review with variation between treatment protocols. However, to reduce the heterogeneity of the study population, we excluded patients who did not receive adjuvant therapy, despite a positive lymph node status. Most adjuvant regimens consisted of FOLFOX (infusion of 5-fluorouracil, leucovorin, and oxaliplatin). Second, the duration of follow-up was relatively short. However, a recent analysis showed that most relapses occur within 2 years of surgery. This study suggested that disease-free survival after 2 and 3 years of follow-up is an appropriate end point for clinical trials in the adjuvant setting[26]. Third, immunohistochemical information on glucose metabolism in colorectal cancer was unavailable. Therefore, a biological explanation for the correlation between FDG accumulation and the behavior of the tumor was not suggested.

In conclusion, our study suggests that high FDG uptake of the primary mass in resectable colorectal cancer is not associated significantly with tumor recurrence or disease-free survival.

COMMENTS

Background

Despite improvement in diagnosis and treatment modalities, mortality from colon cancer has decreased slightly over the past 30 years. Therefore, methods for identifying patients at high risk of recurrence could influence posttreatment surveillance with the ultimate goal of improving survival. Fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET) is a functional imaging method for measurement of tumor glucose use. Some researchers have suggested that metabolic activity on FDG-PET may be a predictive marker for some types of malignancies. However, the prognostic value of FDG-PET/CT for colorectal cancer is less well studied.

Research frontiers

FDG-PET/CT is a molecular imaging tool that monitors tissue glucose metabolism. That cellular glucose metabolism may be correlated with tumor growth and aggressiveness has been suggested in some studies. The biologic correlations of FDG accumulation are less well studied for colorectal cancer than for other cancers. Therefore, in this study, authors evaluated FDG-PET/CT imaging of patients with resectable colorectal cancer and determined whether metabolic activity is correlated with recurrence and disease-free survival.

Innovations and breakthroughs

Several studies have found that metabolic activity on FDG-PET/CT is correlated with tumor biology and survival in colon cancer. However, these studies were limited by experimental studies, small numbers of patients, and one report focused on stage Ⅳ colorectal cancer. This study focused on the role of FDG-PET/CT in providing prognostic information on patients with resectable colorectal cancer. Authors included only resectable colorectal cancers, in which clinicians are most interested. Univariate and multivariate analysis did not provide evidence that disease-free survival rates for the subgroups defined by the median SUVmax were significantly different.

Applications

High FDG uptake of primary mass in resectable colorectal cancer doesn't have a significant relationship with tumor recurrence and disease-free survival.

Peer review

This manuscript evaluate the prognostic value of preoperative FDG-PET/CT in patients with resectable colorectal cancer. This study suggests that the high FDG uptake of primary mass in resectable colorectal cancer doesn't have a significant relationship with tumor recurrence and disease-free survival. The results demonstrate evidence can be considerate by clinicians.

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BRIEF ARTICLE

Predictors of the outcomes of acute-on-chronic hepatitis B liver failure

Hsiu-Lung Fan, Po-Sheng Yang, Hui-Wei Chen, Teng-Wei Chen, De-Chuan Chan, Chi-Hong Chu, Jyh-Cherng Yu, Shih-Ming Kuo, Chung-Bao Hsieh

Hsiu-Lung Fan, Teng-Wei Chen, De-Chuan Chan, Chi-Hong Chu, Jyh-Cherng Yu, Chung-Bao Hsieh, Division of General Surgery, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei 11490, Taiwan, China
Po-Sheng Yang, Division of General Surgery, Department of Surgery, Mackay Memorial Hospital, Taipei 11490, Taiwan, China
Hui-Wei Chen, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, United States
Shih-Ming Kuo, Division of Pediatric Surgery, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei 11490, Taiwan, China
Author contributions: Fan HL analyzed the data and wrote the paper; Yang PS collected the data at Mackay Memorial Hospital; Chen HW collected the data; Chen TW, Chan DC, Chu CH, Yu JC, and Kuo SM determined the direction of this manuscript; Hsieh CB designed the study.
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Correspondence to: Chung-Bao Hsieh, Associate Professor, Division of General Surgery, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, 325, Sec. 2, Chen-Kung Rd, Neihu, Taipei 11490, Taiwan, China. albert0920@yahoo.com.tw
Telephone: +882-2-87923311 Fax: +882-2-87927372
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Abstract

AIM: To identify the risk factors in predicting the outcome of acute-on-chronic hepatitis B liver failure patients.

METHODS: We retrospectively divided 113 patients with acute-on-chronic liver failure-hepatitis B virus (ACLF-HBV) and without concurrent hepatitis C or D virus infection and hepatocellular carcinoma into two groups according to their outcomes after anti-HBV therapy. Their demographic, clinical, and biochemical data on the day of diagnosis and after the first week of treatment were analyzed using the Mann-Whitney *U* test, Fisher's exact test, and a multiple logistic regression analysis.

RESULTS: The study included 113 patients (87 men and 26 women) with a mean age of 49.84 years. Fifty-two patients survived, and 61 patients died. Liver failure (85.2%), sepsis (34.4%), and multiple organ failure (39.3%) were the main causes of death. Multivariate analyses showed that Acute Physiology and Chronic Health Evaluation (APACHE) Ⅱ scores ⩾ 12 [odds ratio (OR) = 7.160, 95% CI: 2.834-18.092, $P < 0.001$] and positive blood culture (OR = 13.520, 95% CI: 2.740-66.721, $P = 0.001$) on the day of diagnosis and model for end-stage liver disease (MELD) scores ⩾ 28 (OR = 8.182, 95% CI: 1.884-35.527, $P = 0.005$) after the first week of treatment were independent predictors of mortality.

CONCLUSION: APACHE Ⅱ scores on the day of diagnosis and MELD scores after the first week of anti-HBV therapy are feasible predictors of outcome in ACLF-HBV patients.

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Key words: Lamivudine; Liver failure; Hepatitis B virus; Acute Physiology and Chronic Health Evaluation Ⅱ score; Model for end-stage liver disease scores

Peer reviewer: Dr. Shashi Bala, Department of Medicine, Umass Medical school, 364 Plantation Street, LRB2701, Worcester, MA 01605, United States

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major global health problem, with higher prevalence in Asia than in the West. In Taiwan, its prevalence is estimated at more than 8%[1]. Approximately 15%-40% of the patients with HBV infection eventually develop cirrhosis, hepatocellular carcinoma, or liver failure[2]. Acute-on-chronic liver failure (ACLF) is characterized by an acute deterioration in liver function due to a precipitating event such as infection, sepsis, alcohol, or variceal bleeding, in a patient with previously chronic liver disease. The reactivation of HBV infection plays an important role in the progression of ACLF in the Asian region[3]. Therefore, the first goal of treatment is to inhibit HBV replication.

Lamivudine, an L-nucleoside analog, has good therapeutic efficacy for suppressing HBV replication[4,5]. Consciousness and coagulopathy recover within only one week of lamivudine therapy, and the response rate to treatment is 86.66%[6]. Lamivudine therapy prevents the progression from acute exacerbation of hepatitis B to liver failure[6]. However, one report has indicated that lamivudine has no overall survival benefit in the management of severe flare-ups of chronic hepatitis B (CHB) with jaundice[7]. Therefore, patients unresponsive to anti-HBV therapy require alternative and effective treatments.

Liver transplantation (LT) is widely performed in patients with fulminant hepatitis B[8,9]. Advances in the transplantation techniques have resulted in good outcomes after living donor liver transplantation (LDLT) or dual-graft LDLT in patients with ACLF-HBV[9,10]. It is important for the transplant surgeon to know the optimal timing and the criteria for LT in patients with ACLF-HBV.

Because both anti-HBV therapy and LT are suitable treatments for ACLF-HBV, hepatologists and transplant surgeons require a practical model to predict the outcomes of ACLF-HBV. The model for end-stage liver disease (MELD) is one such prognostic model[11,12]; however, MELD scores alone do not predict the prognosis accurately[13], and their suitability as prognostic indicators is controversial. Acute Physiology and Chronic Health Evaluation Ⅱ (APACHE Ⅱ) scores are widely used in intensive care units to assess the severity of disease. Although this scoring system has been investigated with respect to its utility in predicting outcomes in critically ill patients with cirrhosis, the suitability of APACHE Ⅱ scores as prognostic indicators for ALCF is also controversial[14-16]. In addition, APACHE Ⅱ scores have rarely been evaluated for predicting the outcomes of acute hepatic failure[17-19].

Previous studies have focused on various risk factors of mortality on the day of diagnosis, reflecting the severity of disease only at the time of diagnosis. ACLF-HBV has a rapidly progressing clinical course. Therefore, prompt evaluation of the efficacy of anti-HBV or supportive treatment would have a great impact on the outcomes of this disease. Accordingly, we aimed to assess the applicability of MELD and APACHE Ⅱ scores as predictors of the outcomes of ACLF-HBV and develop predictive models of mortality from ACLF-HBV based on the clinical and biochemical data recorded on the day of diagnosis and after the first week of anti-HBV and supportive treatment.

MATERIALS AND METHODS

Patients and study design

Between January 2001 and June 2010, 113 patients were diagnosed with ACLF-HBV at these two institutions in Taiwan. ACLF-HBV was defined as the recent development of jaundice (serum total bilirubin level ≥ 10 mg/dL), with an international normalized ratio (INR) > 1.5, and complicated with ascites and/or encephalopathy in a patient previously diagnosed or underdiagnosed with CHB[18]. The definition of underdiagnosed CHB was that HBV tests were performed for the first time and positive in patients with cirrhosis. The exclusion criteria were concurrent hepatitis C or D virus infection, a history of alcoholic drinking, malignant jaundice, hemolytic jaundice, prolonged prothrombin time induced by blood system disease, Wilson disease, autoimmune hepatitis, and anti-HBV therapy for liver failure diagnosed at another hospital.

All the enrolled patients received 100 mg lamivudine (tablets) once daily. Their medical records were retrospectively reviewed, and they were followed up for at least 3 mo. The outcome of each patient was recorded, and the causes of death were documented.

Observed parameters

The results of blood tests performed on the day of diagnosis and after the first week of anti-HBV therapy were recorded. The blood tests performed included white blood count (WBC), hemoglobin level, platelet count, prothrombin time (PT), INR, creatinine level, aspartate, alanine transaminase (ALT) level, total bilirubin (TBil) level, direct bilirubin (DBil) level, total cholesterol level, triglyceride level, albumin level, ammonia level, serum sodium level, serum potassium level, serum chloride level, total serum calcium level, and serum magnesium level.

MELD scores were calculated according to the following formula: MELD score = [3.78 × $\log_e$(bilirubin level in mg/dL)] + [11.2 × $\log_e$(INR)] + [9.6 × $\log_e$(creatinine level in mg/dL)] + [6.4 × (etiology: 0 if cholestatic or alcoholic, 1 otherwise)][20]. APACHE Ⅱ scores were calculated from 12 physiological and laboratory values: temperature, mean arterial pressure, heart rate, respiratory rate, oxygenation (PaO_2 or A-aDo_2), arterial pH, serum sodium level, serum potassium level, serum creatinine level, hematocrit, WBC count, and Glasgow coma score. Each value was marked from 0 to 4, where 0 = normal and 4 = most abnormal. The sum of these values was adjusted according to the patient's age and chronic health problems[21].

Table 1 Patient characteristics and blood tests on the day of diagnosis

	Survival	Dead	*P* value
Case number	52	61	
Age (yr)[1]	43.00 (17.00)	55.00 (19.00)	< 0.001
BMI[1]	25.15 (5.54)	23.41 (6.35)	0.668
Gender: Male	42 (80.8%)	45 (73.8%)	0.378
Past history: Yes			
Diabetes mellitus[2]	3 (5.8%)	18 (29.5%)	0.001
Hypertension[2]	9 (17.3%)	20 (32.8%)	0.060
Coronary artery disease[3]	1 (1.9%)	6 (9.8%)	0.122
Cirrhosis[2]	34 (65.4%)	45 (73.8%)	0.333
Blood culture: Positive[2]	2 (3.8%)	22 (36.1%)	< 0.001
Gastroenteric tract bleeding[2]	8 (15.4%)	18 (29.5%)	0.075
Laboratory investigation			
WBC count (×10^3/cumm)[1]	7.00 (3.60)	7.10 (6.55)	0.668
Hemoglobin (g/dL)[1]	11.80 (4.90)	10.70 (3.85)	0.879
Platelet (×10^3/cumm)[1]	91.00 (108.00)	89.50 (92.50)	0.826
Prothrombin time (s)[1]	18.00 (4.47)	19.90 (7.45)	0.060
INR[1]	2.23 (1.08)	2.66 (1.51)	0.084
Creatinine (mg/dL)[1]	0.80 (0.70)	0.80 (1.00)	0.109
ALT (U/L)[1]	71.00 (1091.00)	180.00 (833.50)	0.250
Total bilirubin (mg/dL)[1]	15.35 (9.65)	13.95 (12.20)	0.425
Direct bilirubin (mg/dL)[1]	7.50 (7.60)	7.50 (9.40)	0.642
Cholesterol (mg/dL)[1]	108.00 (64.50)	104.00 (52.00)	0.208
Triglyceride (mg/dL)[1]	99.00 (62.00)	94.00 (62.00)	0.164
Albumin (g/dL)[1]	2.90 (0.80)	2.60 (0.88)	0.386
Ammonia (μg/dL)[1]	124.00 (75.50)	138.00 (159.00)	0.133
Serum sodium (mmol/L)[1]	135.00 (6.50)	135.00 (7.50)	0.739
Serum potassium (mmol/L)[1]	3.70 (1.02)	4.10 (1.15)	0.008
Serum chloride (mmol/L)[1]	105.00 (9.00)	106.00 (9.00)	0.207
Serum calcium (mg/dL)[1]	8.30 (0.70)	8.10 (0.80)	0.517
Serum magnesium (mg/dL)[1]	2.00 (0.55)	2.00 (0.67)	0.670
MELD score[1]	26.00 (6.00)	29.00 (10.00)	0.091
APACHE-Ⅱ score[1]	12.00 (5.00)	14.00 (3.00)	< 0.001

P values were derived from [1]the Mann-Whitney *U* test, [2]the χ^2 test, and [3]Fisher's exact test. The median (interquartile range) is presented for continuous variables and the number (percentage) is presented for categorical variables. MELD: Model for end-stage liver disease; APACHE-Ⅱ: Acute Physiology and Chronic Health Evaluation Ⅱ; INR: International normalized ratio; ALT: Alanine transaminase; WBC: White blood cell.

Statistical analysis

Unless otherwise stated, continuous variables are presented as the median (interquartile range), and categorical variables are expressed as the number (percentage) of events. To detect differences between the groups, the Mann-Whitney *U* test was used for continuous variables, and the χ^2 test was used for categorical variables. If 20% of the cells had expected numbers < 5, Fisher's exact test was used instead of the χ^2 test. The cut-off value of continuous variables with a *P* value < 0.05 in the Mann-Whitney *U* test was determined by finding the point on the receiver operating characteristic curve closest to (0,1)[22]. Statistically significant variables with a *P* value < 0.05 in the univariate analysis were put into a backward multivariate analysis. New models to predict the outcomes were established based on the results of the backward multivariate analysis. All the statistical calculations were performed using SPSS version 15.0 (IBM-SPSS, Inc., Chicago, IL). Significance was defined as $P < 0.05$.

Table 2 Univariate and multivariate analysis of clinical and biochemical data on the day of diagnosis

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age ⩾ 45	6.912 (3.009-15.879)	< 0.001	NI	
Serum potassium ⩾ 4.0	1.994 (0.928-4.284)	0.077	NI	
APACHE-Ⅱ score ⩾ 12	7.167 (3.072-16.724)	< 0.001	7.160 (2.834-18.092)	< 0.001
Blood culture: Positive	14.103 (3.125-63.639)	0.001	13.520 (2.740-66.721)	0.001
Past diabetes history: Positive	6.837 (1.884-24.814)	0.003	NI	

Age and history of diabetes mellitus were not included for multivariate analyses because these parameters had been calculated for the APACHE-Ⅱ score. OR: Odds ratio; APACHE-Ⅱ: Acute Physiology and Chronic Health Evaluation Ⅱ; NI: Not included in model.

RESULTS

Patient characteristics

The study included 113 patients (87 men and 26 women) with a mean age of 49.84 years. The patient characteristics are summarized in Table 1. Overall, 52 (46%) patients survived the anti-HBV therapy, and 61 (54%) patients died during therapy. The patients who died were older (55 years *vs* 43 years, $P < 0.001$), more often had a history of diabetes mellitus (29.5% *vs* 5.8%, $P = 0.004$), and tended to have positive blood cultures (36.1% *vs* 3.8%, $P < 0.001$). The groups showed no significant differences in terms of gender, body mass index, history of hypertension or coronary artery disease. Liver failure (85.2%), sepsis (34.4%), and multiple organ failure (39.3%) were the main causes of death.

Observed parameters on the day of diagnosis

The results of the blood test on the day of diagnosis are shown in Table 1. The patients who died had significantly higher values of serum potassium ($P = 0.008$) than the survivors. The groups showed no significant differences in the other observed parameters. Furthermore, the patients who died had significantly higher APACHE Ⅱ scores than the survivors (14 *vs* 12, $P \leqslant 0.001$), but no significant difference in MELD scores was detected.

A univariate analysis showed that the frequency of age ⩾ 45 years ($P < 0.001$), history of diabetes mellitus ($P = 0.003$), positive blood culture ($P = 0.001$), and APACHE Ⅱ score ⩾ 12 ($P < 0.001$) differed significantly between the groups (Table 2). However, potassium levels ⩾ 4.0 mmol/L were not a significant risk factor for dying based on the results from the univariate analysis. The trend ($P = 0.077$) was included in the multivariate analysis.

The multivariate analysis included all the significant parameters in the univariate analysis except for age, history of diabetes mellitus and serum potassium level,

Table 3 Laboratory tests after the first week of anti-hepatitis B virus therapy

	Survivors (n = 52)	Dead (n = 61)	P value
White blood cell count ($\times10^3$/cumm)	6.50 (3.40)	9.35 (6.95)	< 0.001
Hemoglobin (g/dL)	10.25 (1.95)	0.20 (3.70)	0.780
Platelet ($\times10^3$/cumm)	92.50 (74.50)	79.50 (91.25)	0.356
Prothrombin time (s)	17.20 (4.85)	21.65 (9.35)	< 0.001
INR	1.96 (1.00)	2.87 (2.38)	< 0.001
Creatinine (mg/dL)	0.70 (0.50)	1.10 (1.35)	< 0.001
ALT (U/L)	95.50 (331.25)	115.50 (299.00)	0.238
Total bilirubin (mg/dL)	12.75 (9.60)	20.30 (15.20)	0.003
Direct bilirubin (mg/dL)	7.45 (7.85)	10.30 (8.60)	0.093
Albumin (g/dL)	3.00 (0.70)	2.80 (0.85)	0.098
Ammonia (μg/dL)	118.00 (96.00)	159.50 (223.00)	0.005
Serum sodium (mmol/L)	134.00 (8.00)	138.00 (8.00)	0.003
Serum potassium (mmol/L)	4.10 (0.90)	3.80 (1.00)	0.649
Serum chloride (mmol/L)	104.00 (14.00)	105.00 (18.00)	0.338
Serum calcium (mg/dL)	8.40 (1.25)	8.45 (1.43)	0.922
Serum magnesium (mg/dL)	1.80 (1.45)	2.30 (0.93)	0.216
MELD score	25.00 (7.00)	32.00 (12.00)	< 0.001
APACHE-Ⅱ score	13.00 (10.00)	17.50 (9.00)	0.014

The median (interquartile range) is presented for continuous variables. P values were derived using the Mann-Whitney U test. MELD: Model for end-stage liver disease; APACHE-Ⅱ: Acute Physiology and Chronic Health Evaluation Ⅱ; INR: International normalized ratio; ALT: Alanine transaminase.

because these parameters were calculated as part of the APACHE Ⅱ scores. Multivariate analysis showed that an APACHE Ⅱ score ≥ 12 [odds ratio (OR) = 7.160, 95% CI: 2.834-18.092, P < 0.001] and positive blood culture (OR = 13.520, 95% CI: 2.740-66.721, P = 0.001) were independent predictive factors of mortality in patients with ACLF-HBV (Table 2).

Observed parameters after the first week of anti-HBV therapy

Table 3 shows the results of blood tests performed after the first week of anti-HBV therapy. WBC count, PT, INR, creatinine level, TBil level, ammonia level, serum sodium level, MELD score, and APACHE Ⅱ score showed significant differences, and their cut-off values were determined by using the Shock index. In the univariate analysis, WBC count ≥ 8500/mm^3 (P = 0.001), PT ≥ 19 s (P < 0.002), INR ≥ 2.5 (P = 0.002), creatinine level ≥ 0.9 mg/dL (P < 0.001), TBil level ≥ 15.5 mg/dL (P = 0.001), ammonia level ≥ 166 μg/dL (P = 0.008), serum sodium level ≥ 136 mmol/L (P = 0.006), MELD score ≥ 28 (P < 0.001), and APACHE Ⅱ score ≥ 14 (P = 0.0018) differed significantly between the survivors and the patients who died (Table 4).

The multivariate analysis included all the significant parameters in the univariate analysis except for WBC count and serum sodium level because these parameters were calculated as part of the APACHE Ⅱ scores. INR, TBil level, and creatinine level were calculated as part of the MELD scores. Multivariate analysis showed that MELD scores ≥ 28 (OR = 8.182, 95% CI: 1.884-35.527, P = 0.005) were independent predictive factors of mortality in patients with ACLF-HBV (Table 4).

Table 4 Univariate and multivariate analysis of clinical and biochemical data after the first week of anti-hepatitis B virus therapy

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
White blood cell count ≥ 8.50	5.940 (2.129-16.571)	0.001[a]	NI	
Prothrombin time ≥ 19	4.018 (1.656-9.747)	0.002[a]	1.264 (0.254-6.296)	0.775
INR ≥ 2.5	3.960 (1.635-9.590)	0.002[a]	NI	
Creatinine ≥ 0.9	4.819 (2.090-11.112)	<0.001[a]	NI	
Total bilirubin ≥ 15.5	4.271 (1.878-9.714)	0.001[a]	NI	
Ammonia ≥ 166	4.167 (1.459-11.900)	0.008[a]	3.976 (0.703-22.482)	0.118
Serum sodium ≥ 136	3.479 (1.432-8.448)	0.006[a]	NI	
MELD score ≥ 28	13.000 (4.604-36.711)	<0.001[a]	8.182 (1.884-35.527)	0.005
APACHE-Ⅱ score ≥ 14	2.941 (1.201-7.203)	0.018[a]	1.597 (0.375-6.809)	0.527

The parameters of white blood cell count, serum sodium, INR, total bilirubin and creatinine were not included for multivariate analyses because these parameters had been calculated for the APACHE-Ⅱ score or MELD score. OR: Odds ratio; MELD: Model for end-stage liver disease; APACHE-Ⅱ: Acute Physiology and Chronic Health Evaluation Ⅱ; INR: International normalized ratio; NI: Not included in model. [a]P < 0.05.

DISCUSSION

ACLF-HBV has a poor prognosis and is associated with high mortality. Sun *et al*[13] reported that the 3-mo overall mortality of patients with ACLF-HBV without lamivudine treatment was 57.84%. Despite lamivudine treatment, the prognosis is unsatisfactory with a mortality rate of approximately 50.7%-75.7%[23]. We found that the 3-mo overall mortality was 54.0%, which is consistent with previous reports. These results indicate that mortality from ACLF-HBV depends on multiple factors other than HBV replication. One relevant factor may be physiological changes in these patients. In addition to liver failure (85.2%), sepsis and multiple organ failure were also important causes of death in the present study, responsible for 34.4% and 39.3% of patient deaths, respectively. Both HBV-induced primary injury and cytokine-induced secondary injury confirm that physiological change is equally important to HBV replication in the pathogenesis of ACLF-HBV. Physiological changes other than HBV replication in a patient with ACLF-HBV are highly associated with patient outcomes. HBV load and a decline in HBV DNA load are proven risk factors[23]. Other risk factors of mortality from ACLF-HBV should be investigated.

Determining which method to use for describing the severity of physiological changes or cytokine-induced secondary injury in patients with ACLF-HBV was an issue of concern in this study. APACHE Ⅱ scores are widely used in intensive care units to evaluate the severity and prognosis of a disease[21]. The predictive accuracy of APACHE Ⅱ scores for mortality in patients with acetaminophen-induced acute liver failure has been reported in terms of the area under the receiver operating characteristic (ROC) curve, sensitivity, and specific-

ity (0.77, 67% and 76%, respectively)[24]. We found that APACHE Ⅱ scoring on the day of diagnosis does not exhibit good predictive accuracy. Through multiple logistic regression analysis, an APACHE Ⅱ score ⩾ 12 and positive blood culture on the day of diagnosis were identified as independent predictive factors of mortality. These two factors accurately predicted the outcome of ACLF-HBV patients better than the APACHE-Ⅱ score alone or prior associated studies (predictive accuracy was 76.6%; area under the ROC curve was 0.804). We ignored age and history of diabetes mellitus in the multivariate analysis because they were calculated in the APACHE Ⅱ scores, but we supposed that these parameters play an important role in determining APACHE Ⅱ scores. The identification of positive blood culture as a predictor of mortality suggests that physicians should treat infection in patients with ACLF-HBV aggressively.

MELD scores are widely used to assess the severity of chronic liver disease and indications for LT[24]. Sun *et al*[13] found that MELD scoring alone had lower predictive accuracy than their predictive model. Our results are consistent with those of Sun *et al*[13] and suggest that MELD scoring on the day of diagnosis does not accurately predict mortality from ACLF-HBV. We supposed that MELD scores, which include INR, TBil level, and creatinine level, are not acute-stage indicators. However, the fact that MELD scores ⩾ 28 after 1 wk are strongly associated with mortality implies that after the acute complications of ACLF are treated and HBV is suppressed, liver function again becomes important as a prognostic factor as it is in cirrhosis without ACLF. The change in MELD score may reflect the efficacy of anti-HBV therapy in patients with ACLF-HBV. Our findings also highlight the importance of repeated evaluations after 1 wk of anti-HBV therapy, given the fact that ACLF-HBV progresses rapidly and that disease severity and the patient's condition cannot be evaluated completely on the day of diagnosis. Furthermore, our results suggest that cytokine-induced physiological changes recover after one week of anti-HBV treatment; therefore, progressive liver injury determines the outcome of ACLF-HBV.

The English literature includes few reports on the prognostic factors of ACLF-HBV. Before the lamivudine era, the prognostic model for ACLF-HBV consisted of the presence of hepatorenal syndrome, the presence of liver cirrhosis, the presence of hepatitis B e antigen, low albumin level, and low prothrombin activity[13]. With the development of anti-HBV therapy, the HBV factor has been investigated further. Dai *et al*[25] found that albumin level, INR, and HBV DNA level are good predictors of mortality in patients with CHB-related decompensation treated with lamivudine. Furthermore, Yu *et al*[26] reported that MELD scores of 30 to 40, low HBV load, and rapid declines in HBV DNA load are good predictors of mortality in patients with ACLF-HBV treated with plasma exchange and lamivudine. Similarly, Sun *et al*[23] showed that MELD scores of 20 to 30, low HBV load, and rapid declines in HBV DNA load are good predictors in patients with ACLF-HBV treated with lamivudine. Therefore, HBV replication is the key factor for predicting mortality. We did not analyze HBV factors because of the lack of complete data.

The present study has some limitations. Because of its retrospective nature, some laboratory data, such as lactate acid level and HBV load, could not be collected and analyzed. These data are considered good predictors of the outcomes of ACLF-HBV[23]. In addition, the results need to be interpreted cautiously, given the differences in the definition of ACLF-HBV between this and previous studies. For example, the plasma TBil level ranged from ⩾ 5 mg/dL to ⩾ 10 mg/dL, which define the jaundice of liver failure in acute-on-chronic liver failure in previous studies; we adopted a plasma TBil level ⩾ 10 mg/dL, which seems to be the most common criterion[13,23,25]. However, the Asian Pacific Association for the Study of Liver held a meeting in 2008, where liver failure in acute-on-chronic liver failure was defined as jaundice (serum bilirubin ⩾ 5 mg/dL) and coagulopathy (INR ⩾ 1.5). In the future, researchers must follow the above definition to help clinicians improve the identification and management of these patients[3].

In conclusion, our logistic regression models have good predictive accuracy. APACHE Ⅱ score ⩾ 12 and positive blood culture on the day of diagnosis reflect severe liver failure. Anti-HBV medication, aggressive supportive treatment, and the control of infection are important for patients with ACLF-HBV. The need for appropriate donors among relatives, the evaluation of liver structures, and the ethical issues inherent in LT should be considered. Patients should be re-evaluated after the first week of anti-HBV treatment. MELD scores ⩾ 28 indicate that the patient is in poor condition. These patients should undergo LT as soon as possible. This study emphasized the selection of an APACHE Ⅱ score and a MELD score as predictors because these two scores are used clinically in many institutions and utilized easily by clinicians. Future research efforts should verify the outcome of liver transplantation for these ACLF-HBV patients with the risk factors described above.

COMMENTS

Background

Acute-on-chronic hepatitis B liver failure has a poor prognosis with high mortality. Despite lamivudine treatment, the prognosis in still unsatisfactory. Acute-on-chronic hepatitis B liver failure has a rapidly progressing clinical course. Prompt evaluation of the efficacy of anti-hepatitis B virus (HBV) treatment would have a great impact on patient outcomes.

Research frontiers

To assess the applicability of Acute Physiology and Chronic Health Evaluation (APACHE)-Ⅱ and model for end-stage liver disease (MELD) scores to predict the outcome of acute-on-chronic hepatitis B liver failure.

Innovations and breakthroughs

The suitability of MELD scores as prognostic indicators for these patients is controversial. APACHE-Ⅱ scores have rarely been evaluated among these patients. Importantly, these two scores are easily available in clinical practice.

Applications

APACHE Ⅱ scores on the day of diagnosis and MELD scores after the first

week of anti-HBV therapy are feasible predictors in determining the outcome of acute-on-chronic hepatitis B liver failure even after treatment with anti-hepatitis B virus therapy.

Terminology

Acute-on-chronic liver failure is characterized by an acute deterioration in liver function as a result of a precipitating event such as infection, sepsis, alcohol toxicity, or variceal bleeding, in a patient with previously chronic liver disease.

Peer review

In this article, the authors have compared various parameters to predict the outcomes of acute-on-chronic hepatitis B liver failure. The immediate message of the conclusion is that we apparently have two concrete, bedside early prognostic indicators. The authors have included the limitations of the presented study.

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BRIEF ARTICLE

Decreased mitochondrial deoxyribonucleic acid and increased oxidative damage in chronic hepatitis C

Hsu-Heng Yen, Kai-Lun Shih, Ta-Tsung Lin, Wei-Wen Su, Maw-Soan Soon, Chin-San Liu

Hsu-Heng Yen, Kai-Lun Shih, Wei-Wen Su, Maw-Soan Soon, Department of Gastroenterology and Hepatology, Changhua Christian Hospital, Changhua 500, Taiwan, China
Ta-Tsung Lin, Chin-San Liu, Vascular and Genomic Research Center, Changhua Christian Hospital, Changhua 500, Taiwan, China
Author contributions: Yen HH and Liu CS contributed equally to this manuscript; Yen HH and Liu CS designed the study and are equally responsible for the drafting, reading and final approval of the manuscript; Shih KL, Su WW and Soon MS participated in the study design, drafting, reading and final approval of the manuscript; Lin TT and Liu CS performed the laboratory study and participated in the data analysis and final approval of the manuscript.
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Correspondence to: Hsu-Heng Yen, MD, Department of Gastroenterology and Hepatology, Changhua Christian Hospital, 135 Nanhsiao Street, Changhua 500, Taiwan, China. 91646@cch.org.tw
Telephone: +886-4-7238595 Fax: +886-4-7228289
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Abstract

AIM: To determine whether alteration of the mitochondria DNA (mtDNA) copy number and its oxidative damage index (mtDNA$^{\Delta CT}$) can be detected by analysis of peripheral blood cells in hepatitis C virus (HCV)-infected patients.

METHODS: This study enrolled two groups of patients aged 40-60 years: a control group and an HCV-infected group in Department of Gastroenterology and Hepatology in Changhua Christian Hospital. Patients with co-infection with hepatitis B virus or human immunodeficiency virus, autoimmune disease, malignant neoplasia, pregnancy, thyroid disease, or alcohol consumption > 40 g/d were excluded. HCV-infected patients who met the following criteria were included: (1) positive HCV antibodies for > 6 mo; (2) alanine aminotransferase (ALT) levels more than twice the upper limit of normal on at least two occasions during the past 6 mo; and (3) histological fibrosis stage higher than F1. The mtDNA copy number and oxidative damage index of HCV mtDNA (mtDNA$^{\Delta CT}$) were measured in peripheral blood leukocytes. The association between mtDNA copy number and mtDNA$^{\Delta CT}$ was further analyzed using clinical data.

RESULTS: Forty-seven normal controls (male/female: 26/21, mean age 50.51 ± 6.15 years) and 132 HCV-infected patients (male/female: 76/61, mean age 51.65 ± 5.50 years) were included in the study. The genotypes of HCV-infected patients include type 1a (n = 3), type 1b (n = 83), type 2a (n = 32), and type 2b (n = 14). Liver fibrosis stages were distributed as follows: F1/F2/F3/F4 = 1/61/45/25 and activity scores were A0/A1/A2/A3 = 7/45/55/25. There were no age or gender differences between the two groups. HCV-infected patients had higher hepatitis activity (aspartate transaminase levels 108.77 ± 60.73 *vs* 23.19 ± 5.47, $P < 0.01$; ALT levels 168.69 ± 93.12 *vs* 23.15 ± 9.45, $P < 0.01$) and lower platelet count (170.40 ± 58.00 *vs* 251.24 ± 63.42, $P < 0.01$) than controls. The mtDNA copy number was lower in HCV-infected patients than in controls (173.49 *vs* 247.93, $P < 0.05$). The mtDNA$^{\Delta CT}$ was higher in HCV-infected patients than in controls (2.92 *vs* 0.64, $P < 0.05$). To clarify the clinical significance of these results in HCV-infected patients, their association with different clinical parameters among HCV-infected patients was analyzed. A negative association was found between mtDNA copy number and elevated aspartate transaminase levels (r = -0.17, $P < 0.05$). Changes in mtDNA copy number were not associated with HCV RNA levels, HCV genotypes, liver fibrosis severity, or inflammatory activity in the liver biopsy specimen. However, a correlation was observed between mtDNA$^{\Delta CT}$ and platelet count (r = -0.22, $P < 0.01$), HCV RNA level (r = 0.36, $P < 0.01$), and hepatitis activity (r = 0.20, P = 0.02). However, no difference in the change in mtDNA$^{\Delta CT}$ was observed between different fibrosis stages or HCV

genotypes.

CONCLUSION: Oxidative stress and mtDNA damage are detectable in patient's peripheral leukocytes. Increased leukocyte mtDNA$^{\Delta CT}$ correlates with higher HCV viremia, increased hepatitis activity, and lower platelet count.

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Key words: Hepatitis C; Mitochondria; Oxidative stress; Mitochondrial DNA; Biomarker

Peer reviewers: Fumio Imazeki, MD, Department of Medicine and Clinical Oncology, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan; Akihito Tsubota, Assistant Professor, Institute of Clinical Medicine and Research , Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan; Hisato Nakajima, MD, Department of Gastroenterology and Hepatology, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan

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INTRODUCTION

Mitochondria play an important role in energy metabolism of cells[1,2]. Human mitochondrial DNA (mtDNA) is prone to oxidative injury because mitochondria generate reactive oxygen species during adenosine triphosphate (ATP) synthesis[3]. Unlike genomic DNA, mitochondria are unique in that they possess multiple copies of mtDNA. Alteration or depletion of mtDNA copy number in tissues or serum has been linked to various diseases[4-7] associated with different oxidative stress conditions.

Hepatitis C virus (HCV) is an important cause of chronic viral hepatitis. Without treatment, patients progress to cirrhosis and eventually develop hepatocellular carcinoma. Several mechanisms have been postulated for HCV-induced liver injury, including immunological hepatocellular damage, cytotoxicity of different viral products, and increased oxidative stress[8]. In previous studies, HCV infection (especially by HCV genotype 1) has also been found to be associated with decreased hepatocyte mtDNA[9]. Some studies suggested hepatocyte mtDNA depletion in HCV/human immunodeficiency virus (HIV) co-infected patients[7,10-12]. However, these studies were limited to a small number of cases and did not include a group of normal controls. Alterations of mtDNA and oxidative stress in HCV-infected patients have been mainly studied in tissues; however, recent observations suggested that use of peripheral blood cells may also be informative[4,5]. Cardin *et al*[13] observed increased oxidative DNA damage in peripheral lymphocytes of HCV-infected patients, and Levent *et al*[8] observed increased oxidative stress in erythrocytes of chronic HCV-infected patients. Thus, we hypothesized that alteration of mtDNA copy number and oxidative damage of mtDNA may be detected by analyzing peripheral blood leukocytes of chronic HCV-infected patients.

MATERIALS AND METHODS

Subjects

The control group comprised 47 healthy persons aged 40-60 years who had been included in our previous study of mtDNA copy number in human leukocytes[5]. The 132 HCV-infected patients included in this study were aged 40-60 years, had undergone liver biopsy, and had received interferon (IFN)-based therapy at the Department of Gastroenterology and Hepatology of Changhua Christian Hospital between October 2003 and July 2009. Exclusion criteria were coinfection with hepatitis B virus or HIV, autoimmune disease, malignant neoplasia, pregnancy, thyroid disease, alcohol consumption > 40 g/d, intravenous drug abuse, and any contraindication for pegylated IFN-α or ribavirin therapy. The study was approved by the Institutional Review Board of Changhua Christian Hospital and conducted in accordance with institutional guidelines.

Criteria for chronic hepatitis C that required combined IFN-based therapy were as follows: (1) patients demonstrating positive HCV antibodies for > 6 mo; (2) patients with alanine aminotransferase (ALT) levels more than twice the upper limit of normal on at least two occasions during the 6 mo prior to enrolment into the study; and (3) patients with a histological fibrosis stage of more than F1 (F0: no fibrosis; F1: portal fibrosis without septa; F2: portal fibrosis with rare septa; F3: numerous septa without cirrhosis; and F4: liver cirrhosis) in the METAVIR scoring system[14].

Assessment of medical history and laboratory tests

Any history of hypertension, diabetes, and hyperlipidemia was recorded. Hypertension was defined as systolic blood pressure > 140 mmHg or use of antihypertensive drugs; diabetes mellitus as hemoglobin A1c > 6.5% or use of oral antidiabetic agents/insulin injection; and hyperlipidemia as total cholesterol level of > 200 mg/dL, low-density lipoprotein cholesterol level of > 130 mg/dL, or triglyceride level of > 200 mg/dL.

Biochemical and hematological tests were performed prior to IFN therapy using commercially available assays. HCV genotype was assigned at baseline by sequencing using a Beckman CEQ 8000 Sequencer (Beckman Coulter, Fullerton, CA, United States). Serum HCV RNA was measured using a quantitative assay (COBAS AMPLICOR Hepatitis C Virus Test, version 2.0; Roche, Branchburg, NJ, United States) with a detection limit of 50 IU/mL. The degree of liver fibrosis (F0: no fibrosis; F1: portal fibrosis without septa; F2: portal fibrosis with

Table 1 Clinical features of normal controls and hepatitis C virus-infected patients (mean ± SD)

	Normal (n = 47)	HCV infection (n = 132)	P value
Age (yr)	50.51 ± 6.15	51.65 ± 5.50	0.24
Gender (male/female)	26/21	71/61	0.99
Smoking (n)	9	28	0.93
Hypertension (n)	1	30	< 0.01
Hyperlipidemia (n)	4	21	0.31
DM (n)	0	35	< 0.01
WBC (10^3/mL)	5.82 ± 1.48	5.75 ± 1.57	0.85
Hb (g/dL)	13.67 ± 1.77	14.49 ± 1.71	0.75
Platelet (10^3/mL)	251.24 ± 63.42	170.40 ± 58.00	< 0.01
AST (IU/L)	23.19 ± 5.47	108.77 ± 60.73	< 0.001
ALT (IU/L)	23.15 ± 9.45	168.69 ± 93.12	< 0.001
mtDNA	247.93 ± 135.46	173.49 ± 147.40	< 0.01
mtDNA$^{\Delta CT}$	0.67 ± 0.48	2.92 ± 2.50	< 0.01

HCV: Hepatitis C virus; ALT: Alanine aminotransferase; AST: Aspartate transaminase; DM: Diabetes mellitus; Hb: Hemoglobin; WBC: White blood cell; mtDNA: Mitochondrial DNA.

rare septa; F3: numerous septa without cirrhosis; and F4: liver cirrhosis) and the intensity of liver necroinflammation (A0: no activity, A1: mild activity, A2: moderate activity, and A3: severe activity) were graded according to the METAVIR scoring system[14] by a single expert pathologist.

Determination of total copy number and oxidative damage in leukocyte mtDNA

Leukocytes were separated from the blood sample taken prior to IFN therapy, and DNA was isolated as described previously[5]. Fluorescence-based quantitative polymerase chain reaction (FQ-PCR) was performed using the LightCycler FastStart DNA Master Kit (Roche Molecular Biochemicals, Penzberg, Germany) to measure total copy number in leukocyte mtDNA[15]. In brief, the threshold cycle number of mitochondrial ND1 and β-globin genes was determined in leukocytes of HCV-infected patients. mtDNA copy number was calculated according to a previously described method[5]. The total amount of mtDNA in leukocytes was expressed as copy number/cell. In addition, the mtDNA oxidative damage index (mtDNA$^{\Delta CT}$) was evaluated as described previously[15]. In brief, human 8-oxoguanine DNA glycosylase (hOGG1) was used to remove 8-hydroxydeoxyguanosine (8-OHdG) from the damaged DNA template in which lesion sites were formed. Amplification in FQ-PCR was then blocked, and the cycle threshold (Ct) value increased. Thus, ΔCt value was determined by calculating the difference between values before and after treatment with hOGG1. A higher mtDNA$^{\Delta CT}$ value indicated the presence of more 8-OHdG in mtDNA, and thus greater oxidative damage.

Statistical analysis

Continuous variables in the groups were compared using the Student's t test or Mann-Whitney U test as appropriate. Categorical variables were evaluated using the χ^2 test.

Table 2 Correlation between mitochondrial DNA and clinical findings in hepatitis C virus-infected patients

Characteristics	Correlation statistics	
	r	P value
Age (yr)	-0.07	0.40
Laboratory data		
AST (IU/L)	-0.17	< 0.05
ALT (IU/L)	-0.07	0.38
Hb (g/dL)	0.14	0.09
Platelet count (10^3/mL)	0.03	0.71
WBC count (10^3/mL)	0.08	0.34
HCV RNA	-0.10	0.47
Liver histology		
Inflammatory activity	-0.06	0.45
Fibrosis staging	0.04	0.59

HCV: Hepatitis C virus; ALT: Alanine aminotransferase; AST: Aspartate transaminase; Hb: Hemoglobin; WBC: White blood cell.

Relationships between two continuous variables were evaluated by Pearson correlation. Differences between the two groups were considered significant when the P value was < 0.05. Statistical analyses were performed using MedCalc software version 11.5 (MedCalc Software bvba, Broekstraat 52, 9030 Mariakerke, Belgium).

RESULTS

Controls and HCV-infected patients

Clinical features of the controls and HCV-infected patients are shown in Table 1. Genotypes of HCV-infected patients include type 1a (n = 3), type 1b (n = 83), type 2a (n = 32), and type 2b (n = 14). The distribution of liver fibrosis stages was as follows: F1/F2/F3/F4 (n = 1/61/45/25) and activity score A0/A1/A2/A3 (n = 7/45/55/25). A negative association (r = -0.2277, P < 0.01) was found between mtDNA content and the extent of mtDNA oxidative stress (measured as mtDNA$^{\Delta CT}$) (Figure 1). There were no age or gender differences between the patient and control groups. A lower platelet count and higher hepatitis activity were found in HCV-infected patients (P < 0.01). In addition, the peripheral blood lymphocyte mtDNA copy number in HCV-infected patients was lower (173.5 *vs* 247.93, P < 0.01) and mtDNA$^{\Delta CT}$ was higher (2.92 *vs* 0.67, P < 0.01) than those in the control group.

Alterations in mtDNA copy number in HCV-infected patients

To clarify further the clinical significance of alterations in mtDNA copy number in HCV-infected patients, their association with different clinical parameters was analyzed (Table 2). A negative association was found with elevated aspartate transaminase (AST) levels (r = -0.17, P < 0.05). However, alterations in mtDNA copy number were not associated with the HCV viremia level, HCV genotypes, severity of liver fibrosis, or inflammatory activity in the liver biopsy specimen.

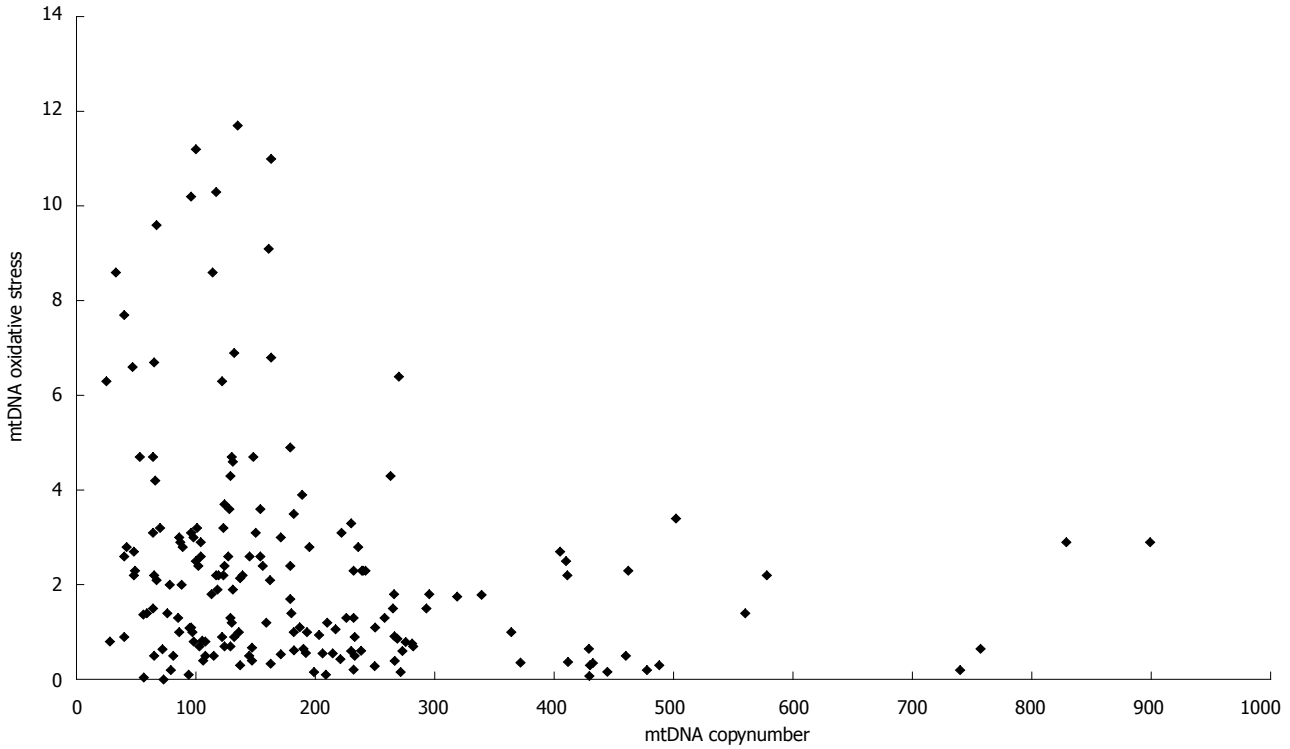


Figure 1 Scatter diagram. A negative correlation is seen between mitochondria DNA (mtDNA) copy number and its oxidative damage index (n = 179, r = -0.24; P < 0.01).

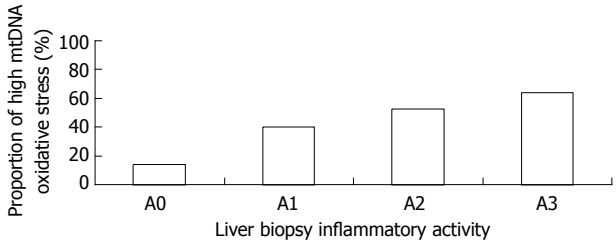


Figure 2 Increased hepatic inflammatory activity is correlated with increased peripheral blood mitochondrial oxidative stress, and mitochondria DNA oxidative damage increased with increased hepatic inflammatory activity (Cochran-Armitage test for trend, P < 0.01). mtDNA: Mitochondria DNA.

Alterations in 8-OHdG content (ΔCt) in HCV-infected patients

To evaluate the clinical significance of alterations in 8-OHdG content in mitochondrial DNA (measured as $mtDNA^{\Delta Ct}$) in HCV-infected patients, their association with different clinical parameters was analyzed (Table 3). Higher $mtDNA^{\Delta Ct}$ suggested higher mitochondrial oxidative stress. A negative association with $mtDNA^{\Delta Ct}$ was observed for decreased platelet count (r = -0.22, P < 0.01). Increased $mtDNA^{\Delta Ct}$ was associated with increased inflammatory activity in liver tissue (r = 0.20, P = 0.02) and the HCV RNA level (r = 0.36, P < 0.01). The trend of mtDNA oxidative damage (low damage: $mtDNA^{\Delta Ct}$ ≤ median $mtDNA^{\Delta Ct}$) increased with increased hepatic inflammatory activity (Cochran-Armitage test for trend, P < 0.01) (Figure 2).

Table 3 Correlation between mitochondria DNA oxidative index and clinical findings in hepatitis C virus-infected patients

Characteristics	Correlation statistics	
	r	*P* value
Age (yr)	0.16	0.06
Laboratory data		
AST (IU/L)	0.15	0.08
ALT (IU/L)	0.09	0.25
Hb (g/dL)	-0.06	0.47
Platelet count (10^3/mL)	-0.22	< 0.01
WBC count (10^3/mL)	-0.13	0.13
HCV RNA	0.36	< 0.01
Liver histology		
Inflammatory activity	0.20	0.02
Fibrosis staging	0.05	0.61

HCV: Hepatitis C virus; ALT: Alanine aminotransferase; AST: Aspartate transaminase; Hb: Hemoglobin; WBC: White blood cell.

DISCUSSION

This was the first study to detect and compare differences in mtDNA copy number and $mtDNA^{\Delta CT}$ between a normal population and HCV-infected patients.

Oxidative stress and mitochondrial injury have been postulated as mechanisms in the pathogenesis of HCV-related liver injury and subsequent development of cirrhosis or hepatocellular carcinoma. Previous studies have suggested that protein products of hepatitis C, for example, core, NS5A, and NS3 proteins, may directly in-

duce oxidative injury in hepatocytes[2,16]. These proteins cause Ca^{2+} uptake into the mitochondria, oxidation of mitochondrial glutathione, and subsequent production of free radicals by the mitochondrial electron transport complex, and induce apoptosis of HCV-infected hepatocytes[2,17]. Consequently, the extent of mitochondrial injury and the severity of oxidative injury exerted in the liver tissue are correlated with the severity of HCV infection[9]. Barbaro *et al*[9] first studied liver biopsy specimens from 75 HCV-infected patients and 22 normal subjects. Hepatic mtDNA levels were decreased in HCV-infected patients, especially in those with HCV genotype 1, with increased free radical production. These findings were also observed in subsequent studies[18,19]. However, direct measurement of such changes in the liver tissue is difficult and invasive, whereas measurements in peripheral blood are more attractive because they are easier and less invasive. HCV is also lymphocytotropic[20], which accounts for the many immunological manifestations in HCV-related disease. Peripheral blood leukocytes can therefore be used as surrogates for hepatocytes.

Cardin *et al*[13] measured 8-OHdG levels as a marker of DNA oxidative damage in circulating leukocytes from 110 HCV-infected patients and 20 normal subjects and found increased levels in the former, showing that increased oxidative DNA can also be detected in peripheral blood leukocytes. In our previous studies[5,21,22], we found that measurement of the peripheral blood mtDNA copy number was a useful marker of oxidative stress and was associated with differences in disease status. The present study suggests that measurement of the peripheral blood leukocyte mtDNA copy number and oxidative stress can be used as a noninvasive marker of mitochondrial damage in HCV-infected patients. Whether or not this information can be utilized as a biomarker to evaluate the activity or severity of HCV-related liver disease remains unclear.

Blood ALT levels and severity of liver inflammation have been found to correlate with 8-OHdG levels in liver tissue, reflecting oxidative DNA damage in hepatocytes[18,19,23]. However, Chuma *et al*[23] found that 8-OHdG levels in serum tended to correlate with those in liver tissue. In the present study, all HCV-infected patients underwent liver biopsy for pretreatment evaluation, thereby facilitating comparison of mtDNA copy number and oxidative stress in different HCV-infected patient groups in terms of the degree of inflammation or fibrosis. The mtDNA copy number was negatively associated with AST levels, but was not correlated with the severity of liver fibrosis or inflammation. In contrast, $mtDNA^{\Delta CT}$ was correlated with inflammatory activity and HCV viremia levels. This finding suggested that $mtDNA^{\Delta CT}$ in peripheral blood leukocytes can be used as a biomarker to evaluate the severity of hepatocyte injury.

This study had several limitations. First, HCV-infected patients eligible for IFN-based treatment were cases with ALT levels two-fold higher than the normal limit or with a biopsy-proven liver fibrosis score more than F1 determined on the basis of national treatment guidelines that were in effect before 2009. Therefore, use of mtDNA copy number and changes in oxidative markers may not be extrapolated to HCV-infected patients with less severe disease (i.e., ALT levels less than two-fold higher than the normal limit or a fibrosis score lower than F1). A recent report[20] suggested that mtDNA damage can be observed even in occult HCV-infected patients. Further studies are still required to evaluate and compare the clinical significance of mtDNA damage between occult and overt HCV-infected patients. Second, subjects in this study were not followed for subsequent measurement of alterations in mtDNA or liver biopsy following IFN-based therapy. Therefore, changes in mtDNA damage over time among different HCV-infected patient groups remain to be investigated. Mutation or depletion of mtDNA has been implicated in development of several cancers[24-26] through changes in the microenvironment of nuclear DNA that lead to progression of cancer[26]. Several reports have suggested that such oxidative DNA damage in the liver tissue can be a risk factor for the subsequent development of hepatocellular carcinoma[4,23,27]. This also supports the finding that antiinflammatory therapy[28,29], in addition to IFN, can decrease the risk of hepatocellular carcinoma. Further studies are still required to evaluate changes in peripheral leukocyte mtDNA damage over time and the risk of the subsequent development of hepatocellular carcinoma.

In conclusion, oxidative stress and mtDNA damage is detectable in peripheral leukocytes of HCV-infected patients. The degree of peripheral leukocyte mtDNA damage correlated with increased hepatic inflammation. However, further studies are required to explore the long-term clinical significance of these findings in HCV patients.

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COMMENTS

Background

The alteration of mitochondria DNA (mtDNA) copy number and its oxidative damage index ($mtDNA^{\Delta CT}$) is associated a different disease status. It is not clear whether such changes can be detected by analysis of peripheral blood cells in hepatitis C virus (HCV)-infected patients.

Research frontiers

Previous studies utilize the liver tissue to evaluate the mitochondria injury in chronic hepatitis C infected patients. In this study, the authors studied the change of mitochondrial injury of peripheral blood cells among HCV-infected patients and normal controls. The authors also use a simplified method to determine the extent of mitochondrial oxidative injury (measured as $mtDNA^{\Delta CT}$) among these two groups of patients. In addition, the authors further study the association of mitochondrial damage with different clinical parameters among HCV-infected patients.

Innovations and breakthroughs

This study aimed to detect and compare differences in mtDNA copy number and $mtDNA^{\Delta CT}$ between a normal population and HCV-infected patients. Oxidative stress and mitochondrial injury have been postulated as mechanisms in the pathogenesis of HCV-related liver injury and subsequent development of cirrhosis or hepatocellular carcinoma. Direct measurement of such changes in the liver tissue is difficult and invasive. In this study, the authors found suggestions

that measurement of the peripheral blood leukocyte mtDNA copy number and oxidative stress can be used as a noninvasive marker of mitochondrial damage in HCV-infected patients.

Applications

The study results suggest that measurement of peripheral blood leukocytes for changes in mitochondrial damage provides an alternative approach to assessing hepatitis C related liver injury.

Peer review

The authors examined the mtDNA copy number and oxidative damage index of mtDNA in the peripheral blood leukocytes of HCV-infected patients in comparison with normal controls. This is an interesting study utilizing unique techniques to determine the copy number of mtDNA and its oxidative damage index. The results are interesting and suggest that oxidative stress and mtDNA damage are detectable in peripheral leukocytes of HCV-infected patients. These findings may lead people to an alternative approach to treatment of HCV infection.

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BRIEF ARTICLE

Impact of body mass index and gender on quality of life in patients with gastroesophageal reflux disease

Shou-Wu Lee, Han-Chung Lien, Chi-Sen Chang, Yen-Chun Peng, Chung-Wang Ko, Ming-Chih Chou

Shou-Wu Lee, Ming-Chih Chou, Institute of Medicine, Chung Shan Medical University, Taichung 40242, Taiwan, China
Shou-Wu Lee, Han-Chung Lien, Chi-Sen Chang, Yen-Chun Peng, Chung-Wang Ko, Division of Gastroenterology, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung 40242, Taiwan, China
Shou-Wu Lee, Chi-Sen Chang, Ming-Chih Chou, Department of Medicine, Chung Shan Medical University, Taichung 40242, Taiwan, China
Han-Chung Lien, Yen-Chun Peng, Chung-Wang Ko, Department of Medicine, China Medical University, Taichung 40242, Taiwan, China
Author contributions: Lee SW and Chou MC contributed equally to this work; Lee SW, Lien HC and Chang CS designed the research; Peng YC and Ko CW performed the research; Lee SW, Lien HC and Chang CS analyzed the data; and Lee SW and Chou MC wrote the paper.
Correspondence to: Dr. Ming-Chih Chou, Department of Medicine, Chung Shan Medical University, 110, Section 1, Chien Kuo North Road, Taichung 40242, Taiwan, China. eric2248836@yahoo.com.tw
Telephone: +886-4-23592525 Fax: +886-4-23741331
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Abstract

AIM: To investigate the symptom presentation and quality of life in obese Chinese patients with gastroesophageal reflux disease (GERD).

METHODS: Data from patients diagnosed with GERD according to the Montreal definition, were collected between January 2009 to March 2010. The enrolled patients were assigned to the normal [body mass index (BMI) < 25 kg/m^2], overweight (25-30 kg/m^2), and obese (BMI > 30 kg/m^2) groups. General demographic data, endoscopic findings, and quality of life of the three groups of patients were analyzed and compared.

RESULTS: Among the 173 enrolled patients, 102, 56 and 15 patients were classified in the normal, overweight, and obese, respectively. There was significantly more erosive esophagitis (73.3% *vs* 64.3% *vs* 39.2%, P = 0.002), hiatal hernia (60% *vs* 33.9% *vs* 16.7%, P = 0.001), and males (73.3% *vs* 73.2% *vs* 32.4%, P = 0.001) in the obese cases. The severity and frequency of heartburn, not acid regurgitation, was positively correlated with BMI, with a significant association in men, but not in women. Obese patients were prone to have low quality of life scores, with obese women having the lowest scores for mental health.

CONCLUSION: In patients with GERD, obese men had the most severe endoscopic and clinical presentation. Obese women had the poorest mental health.

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Key words: Gastroesophageal reflux disease; Gender; Heartburn; Life's quality; Obesity

Peer reviewer: Dr. Wojciech Blonski, MD, PhD, Gastroenterology Unit, University of Pennsylvania, 3400 Spruce St, Philadelphia, PA 19104, United States

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a chronic disease that tends to relapse and develop complications. According to previous studies, the risk factors in patients with GERD include age[1,2], obesity[3,4], hiatal hernia[5,6], and an unfavorable lifestyle, such as alcohol drinking and cigarette smoking. Obesity, defined by a body mass

index (BMI) over 30 kg/m^2, constitutes a public health problem in most industrialized countries, and impairs health-related quality of life[7]. At present, there are few studies on the assessment of quality of life on obese patients with GERD. There might exist a difference in the presentation of symptoms and quality of life between obese and normal weight patients. The aim of the study was to investigate symptom presentation and quality of life in GERD patients with normal or high BMI among a Chinese population in Taiwan.

MATERIALS AND METHODS

Data from consecutive adult patients, older than 18 years, in our hospital, diagnosed with GERD according to the Montreal definition, were collected from January 2009 to March 2010. The Montreal definition of GERD is a condition which develops when the reflux of stomach contents causes troublesome symptoms and/or complications[8]. Exclusion criteria were as follows: (1) GERD combined with other structural gastrointestinal disorders, such as peptic ulcer disease, esophageal or gastric malignancy; (2) prior gastric and esophageal surgery; (3) use of chronic antacid medication, such as proton pump inhibitors or H2-receptor antagonists, for more than 2 mo prior to enrollment; and (4) pregnancy. This study was conducted with the approval of the Clinical Research Ethics Committee of Taichung Veterans General Hospital.

General data of enrolled patients, including age, gender, BMI, and symptom duration, were recorded. All patients underwent an open-access transoral upper gastrointestinal endoscopy, and details of esophagocardiac mucosal break or hiatal hernia in each case were collected.

The patients were stratified into three groups according to BMI: a normal weight group (BMI < 25 kg/m^2), an overweight group (BMI 25-30 kg/m^2), and an obese group (BMI > 30 kg/m^2). All patients were asked to complete the modified Chinese GERD questionnaire (GERDQ) and the short-form 36 (SF-36) questionnaire (Chinese version).

The modified Chinese GERDQ included with the severity and frequency of symptoms of regurgitation and heartburn, graded on a 3-point Likert scale. Mild, moderate and advanced severity were defined, respectively, as symptoms that can easily be ignored, symptoms that can be easily tolerated, and symptoms sufficient to cause interference with normal activities. Low, middle, and high frequency were classified, respectively, as at least once per month, at least once weekly, and at least once daily[9].

The SF-36 questionnaire measures generic quality of life, which allows comparisons between different disease states. It measures health status in eight domains: physical functioning, role limitations-physical, bodily pain, general health, vitality, social functioning, role limitations-emotional, and mental health. Two summary scores are also calculated from subject responses: the physical health (PH) score and the mental health (MH) score. Scores on the SF-36 range from 0 to 100 on each dimension and on the summary scales, with higher scores indicating better quality of life[10,11].

Data are expressed as mean and standard deviation for each of the measured parameters. Gender and endoscopic findings, including hiatal hernia and erosive esophagitis, are expressed as a percentage of the total patient number. A P value < 0.05 was considered statistically significant. Statistical comparisons were made using Pearson's χ^2 test to compare the effects of gender and endoscopic findings; analysis of variance was used to analyze age, symptom duration, and of questionnaire scores.

RESULTS

A total of 173 consecutive patients were enrolled between January 2009 and March 2010, with 102 (58.9%), 56 (32.4%), and 15 patients (8.7%) assigned to the normal weight, overweight, and obese groups, respectively. As shown in Table 1, there were significantly more women in the normal weight group (67.6%), but significantly more men in the overweight (73.2%) and obese (73.3%) groups. A longer mean duration of GERD symptoms was noted in patients with increased BMI (normal weight group 2.17 mo, overweight group 2.64 mo, obese group 3.15 mo, P = 0.406), although the differences were not significant. The mean age of the cases in these three groups were similar.

The endoscopic findings among the three groups are also summarized in Table 1. The more obese patients had a significantly greater rate of erosive esophagitis (73.3% in the obese group, 64.4% in the overweight group, 39.2% in the normal weight group, P = 0.002), and hiatal hernia (60% in the obese group, 33.9% in the overweight group, 16.7% in the normal weight group, P = 0.001). Furthermore, there was a trend for the rate of esophagocardiac ulcer to be increased in obese patients (13.3% in the obese group, 8.9% in the overweight group, 6.9% in the normal weight group, P = 0.666).

The severity and frequency of heartburn and acid regurgitation, measured by the modified Chinese GERDQ, among the three groups are presented in Table 2. There was significantly greater severity and higher frequency of heartburn in obese patients than in those with normal BMI. In contrast, the severity and frequency of acid regurgitation wer similar among these groups.

The total scores, PH scores and MH scores, measured by the SF-36 questionnaire, are shown in Figure 1. The patients in the obese group tended to have lower scores (mean total score 63.64, PH score 63.44, MH score 58.32), than those in the overweight group (mean total score 65.69, PH score 66.90, MH score 60.20) and in the normal group (mean total score 71.47, PH score 69.02, MH score 66.87), but there was no significant difference among these groups (P = 0.351).

Comparing the endoscopic findings by gender, as

Table 1 Basic characteristics of the three groups of patients with gastroesophageal reflux disease

Variable	Normal weight (n = 102)		Overweight (n = 56)		Obese (n = 15)		P value
	n (%)	mean ± SD	n (%)	mean ± SD	n (%)	mean ± SD	
Age (yr)		44.32 ± 15.14		50.77 ± 15.88		41.47 ± 12.24	0.423[2]
Gender							0.001[1]
Male	33 (32.4)		41 (73.2)		11 (73.3)		
Female	69 (67.6)		15 (26.8)		4 (26.7)		
Symptom duration (mo)		2.17 ± 3.16		2.64 ± 3.69		3.15 ± 4.30	0.406[2]
NERD	62 (60.8)		20 (35.7)		4 (26.7)		0.002[1]
EE	40 (39.2)		36 (64.3)		11 (73.3)		
Hiatal hernia	17 (16.7)		19 (33.9)		9 (60.0)		0.001[1]
ECJ ulcer	7 (6.9)		5 (8.9)		2 (13.3)		0.666[1]

[1]Pearson's Chi-square test; [2]Analysis of variance. ECJ: Esophagocardiac junction; EE: Erosive esophagitis; NERD: Non-erosive reflux disease.

Table 2 Basic characteristic of the three groups of male and female patients with gastroesophageal reflux disease

Variable	Normal weight (n = 102)		Overweight (n = 56)		Obesity (n = 15)		P value
	n (%)	mean ± SD	n (%)	mean ± SD	n (%)	mean ± SD	
Male	33 (38.8)		41 (48.3)		11(12.9)		
Mean age (yr)		51.88 ± 16.93		52.54 ± 16.96		39.64 ± 11.09	0.105[2]
NERD	20 (60.6)		10 (24.4)		4 (36.4)		0.007[1]
EE	13 (39.4)		31 (75.6)		7 (63.6)		
Hiatal hernia	10 (30.3)		14 (34.1)		7 (63.6)		0.126[1]
ECJ ulcer	5 (15.2)		5 (12.2)		2 (18.2)		0.859[1]
Female	69 (78.4)		15 (17.1)		4 (4.5)		
Age (yr)		40.71 ± 12.84		45.93 ± 11.59		46.50 ± 15.59	0.248[2]
NERD	42 (60.9)		10 (66.7)		0		0.045[1]
EE	27 (39.1)		5 (33.3)		4 (100)		
Hiatal hernia	7 (10.1)		5 (33.3)		2 (50.0)		0.014[1]
ECJ ulcer	2 (2.9)		0		0		0.754[1]

[1]Pearson's χ^2 test; [2]Analysis of variance. ECJ: Esophagocardiac junction; EE: Erosive esophagitis; NERD: Non-erosive reflux disease.

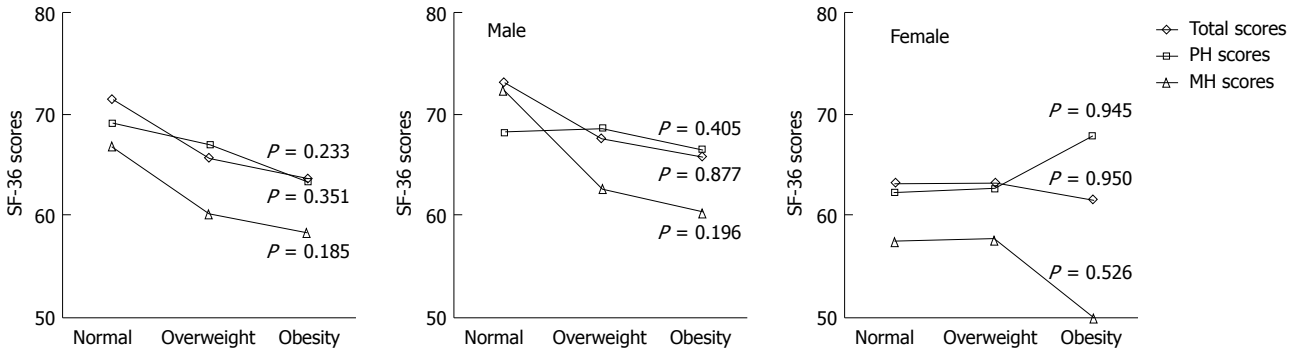


Figure 1 Characteristics of short-form 36 questionnaire scores in obese patients with gastroesophageal reflux disease. SF-36: Short-form 36; PH: Physical health; MH: Mental health.

shown in Table 2, men were more prone to have erosive esophagitis in the overweight (75.6%) and obese (63.6%) groups. In contrast, women had similar rates of erosive eosphagitis, in the normal weight group (39.1%) or the overweight group (33.3%), though all four obese women had erosive esophagitis. The occurrence of hiatal hernia had a significantly positive correlation with obesity in women (P = 0.014), but not in men (P = 0.126).

The severity and frequency of heartburn and acid regurgitation among the three groups of men and women are shown separately in Figure 2. In general, the male cases had more serious symptoms, and there was significantly greater severity and frequency of heartburn in obese males (P = 0.001). In contrast, there were no differences in severity and frequency of heartburn and acid regurgitation among female patients in all groups

The scores of the SF-36 questionnaire in male and female patients are also summarized in Figure 1. The men in the obese group had the lowest scores (mean total score 65.97, PH score 66.33, MH score 60.33), followed

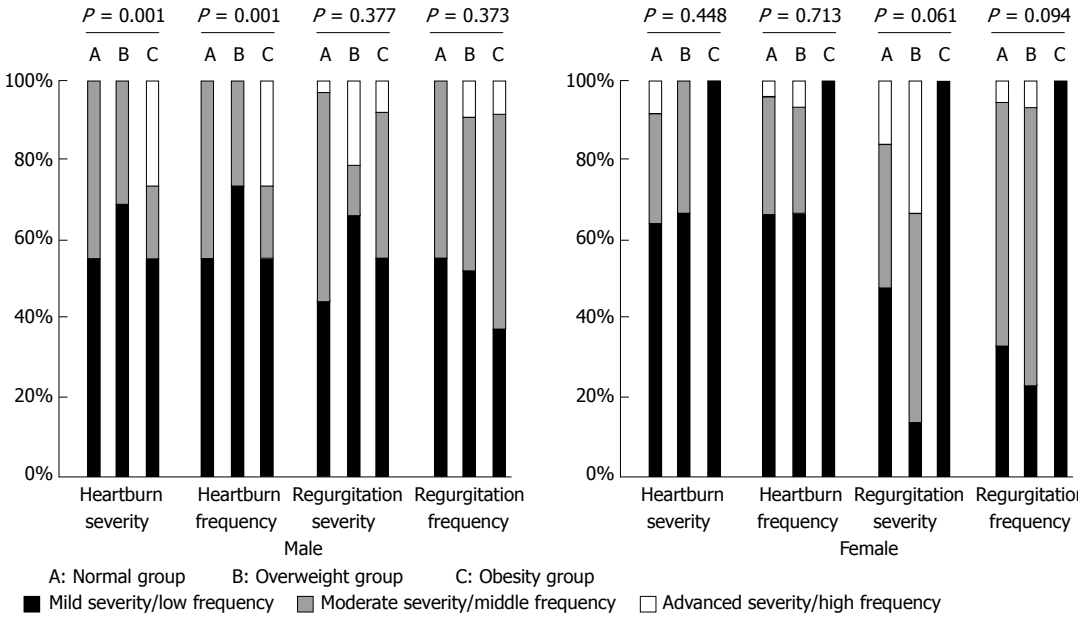


Figure 2 Rates of clinical presentation of symptoms in obese patients with gastroesophageal reflux disease.

by those in the overweight group (mean total score 67.59, PH score 68.61, MH score 62.63) and those in the normal weight group (mean total score 73.27, PH score 68.09, MH score 72.82). In contrast, there were no differences in women's scores among the obese group (mean total score 61.67, PH score 68.02, MH score 49.32), the overweight group (mean total score 63.40, PH score 62.91, MH score 67.81) and the normal weight group (mean total score 63.06, PH score 62.52, MH score 57.59).

DISCUSSION

GERD is a chronic disease which has an impact on the everyday lives of affected individuals, and obesity is found to be a significant risk factor in the development of severe forms of GERD. Changes in gastroesophageal anatomy and physiology caused by obesity may explain the association. Thus, obese patients may experience extrinsic gastric compression by surrounding adipose tissue, and has been associated with significantly increased intra-abdominal pressure[12], impaired gastric emptying[13], decreased lower esophageal sphincter pressure, and increased frequency of transient sphincter relaxation[14,15], leading to increased esophageal acid exposure time[5] .

Obesity is also considered an independent risk factor for the presence of a hiatal hernia[6], which has a role in initiating and promoting GERD. In patients with a hiatal hernia, esophagitis or abnormal low distal esophageal pH were more common than in those without a hiatal hernia[5]. However, after controlling for the presence of hiatal hernia, obesity still remained an independent risk factor for severe esophagitis[3].

In our study, obese patients with GERD had a significantly higher rate of erosive esophagitis and hiatal hernia, but the mean age and GERD symptom duration of these cases were not different. Furthermore, obese men had a more significant association with erosive esophagitis than obese women, but the association of hiatal hernia and obesity was significant only in female patients. Thus, both obesity and hiatal hernia were contributing factors to GERD, but there existed a complicated pathogenesis.

A German study reported that neither the frequency nor the duration of symptoms, including heartburn and acid regurgitation, differed significantly between obese patients and normal body mass cases[16]. Another study reported a positive association between frequency of reflux symptoms and BMI[17]. In addition, an increase in reflux symptoms has been shown to occur in individuals who gain weight, contributing to the epidemiological evidence for a possible dose-response relationship between BMI and increasing GERD symptoms[4].

There were many previous studies discussing the impact of different genders on GERD. One famous study examined a large population-based cohort and found that obese women had an increased risk for GERD symptoms compared with obese men, and the risk was highest in premenopausal women and postmenopausal women receiving estrogen therapy[18]. Other population-based case-control endoscopic studies found a strong and dose-dependent association between BMI and reflux esophagitis, particularly in women rather than men, and hypothesized that this might be caused by increased estrogen activity in overweight and obese females[19,20].

However, several other studies found that the increased risk of acid-related esophageal disease among overweight and obese persons was neither confounded nor modified by gender. For example, a large cross-sectional study found that increased BMI was associated with reflux symptoms, but the relationship differed for different ethnic groups, with a much greater association in whites, but no relationship in the Asian population

in either men or women[21]. A recent systematic review either found no association between gender and GERD symptoms[22]. Thus, the estrogen hypothesis does not explain the gender- or race-related differences in GERD.

Our study documented that both the severity and frequency of heartburn had a positive correlation with BMI, but the symptom of acid regurgitation did not. The relationship was present in male patients, but not in females. A Spanish study in severely obese patients found higher rates of heartburn (46.4%) than acid regurgitation (22.5%), and the frequency of symptoms was higher in men than in women[23], which was similar to our results. The possible explanation of the gender predilection of GERD symptoms in males is that abdominal obesity is characteristic of male pattern obesity, and abdominal obesity is known to be a key factor in both the symptoms and perhaps even the complications of GERD.

The results of our study also found reduced overall quality of life scores, measured by the SF-36 questionnaire, in obese patients with GERD, and the association was observed in male patients, but not in females. Increasing overweight has been associated with a deterioration in quality of life, and the risk of suffering from any longstanding illness is associated with an increased BMI[6]. Obese women perceived markedly more psychosocial problems due to their obesity than men[24]. In our patients, obese women had the lowest MH scores (60.33) among all groups in our study.

There were some limitations in our study. Firstly, the lifestyle characteristics in our study were only limited to the patients' current status, and the past history of each case, such as cigarette smoking and alcohol consumption, was not recorded. Secondly, comorbidity in these patients that tend to influence the severity of GERD, such as chronic heart failure or chronic obstructive pulmonary disease, were not considered, and this might have led to inaccurate outcomes. Thirdly, we only collected the BMI of each patient, but abdominal girth, an indicator of abdominal obesity, might be more correlated with GERD. In addition, a long-term record of BMI would also be necessary to ensure adequate analysis of the relationship of obesity with GERD. Lastly, our study's design was hospital-based and included only a small number of cases. Further research in representative samples of the general population are needed to confirm these results.

In conclusion, in the present study, obese GERD patients were more likely to have higher rates of erosive esophagitis and hiatal hernia, more severe and frequent heartburn symptoms, and a poorer quality of life. Obesity in men was associated with the most severe endoscopic and clinical presentations, but obesity in women had the greatest influence on mental health.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a chronic disease that tends to relapse and develop complications. Obesity constitutes a public health problem in most industrialized countries, and impairs health-related quality of life. At present, there are few studies on the assessment of quality of life performed on obese patients with GERD.

Research frontiers

Body mass index is one risk factor for GERD. The aim of the study was to investigate symptom presentation and quality of life in male and female obese patients with GERD among a Chinese population.

Innovations and breakthroughs

Obese GERD patients had higher rates of erosive esophagitis and hiatal hernia, more severe and frequent heartburn, and poorer quality of life. Obese men had the most severe endoscopic and clinical presentation, while obese women had the poorest mental health.

Applications

The presentations and impact of GERD were apparent in obese patients, but different between men and women. Obese women had the worst quality of life.

Peer review

This is a good descriptive study in which authors investigate the symptom presentation and quality of life in the obese patients with GERD among a Chinese population. The results are interesting and suggest that the obese men owned the most severe endoscopic and clinical presentation, but obese women had the worst mental health.

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BRIEF ARTICLE

Influence of *Helicobacter pylori* infection on ghrelin levels in children

Zhao-Hui Deng, Bo Chu, Ya-Zhen Xu, Bin Zhang, Li-Rong Jiang

Zhao-Hui Deng, Bo Chu, Ya-Zhen Xu, Bin Zhang, Li-Rong Jiang, Department of Pediatrics, Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, Shanghai 200127, China
Author contributions: Deng ZH, Chu B and Xu YZ performed the majority of experiments; Deng ZH was also involved in editing the manuscript; Zhang B analysis the result of experiments; and Jiang LR designed the study and wrote the manuscript.
Supported by Nutrition and Development Center in Shanghai Children's Medical Center
Correspondence to: Dr. Li-Rong Jiang, Department of Pediatrics, Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, Shanghai 200127, China. jiangl_rong@yahoo.com.cn
Telephone: +86-21-38626161 Fax: +86-21-58393915
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Abstract

AIM: To compare ghrelin levels in plasma and gastric mucosa before and after *Helicobacter pylori* (*H. pylori*) treatment in children with *H. pylori*-associated functional dyspepsia.

METHODS: Children with *H. pylori*-associated functional dyspepsia were enrolled in this study. *H. pylori* infection was confirmed by positive bacterial culture results. All of the children received triple *H. pylori* eradication therapy (a 2 wk course of omeprazole, amoxicillin, and clarithromycin). The children were divided into two groups based on the success of the *H. pylori* treatment: group 1 (eradicated) - patients who had a negative 13C-urea breath test 2 mo after the end of therapy; and group 2 (non-eradicated) - patients who had a positive 13C-urea breath test. Plasma ghrelin, gastric ghrelin mRNA, and the body mass index were evaluated in both groups before and after the *H. pylori* treatment. The plasma ghrelin levels were measured by a radioimmunoassay. The expression of gastric ghrelin mRNA was determined by real-time reverse transcription polymerase chain reaction.

RESULTS: A total of 50 children with *H. pylori*-associated functional dyspepsia were treated with triple *H. pylori* eradication therapy. The mean age of the children was 5.52 ± 0.83 years, and there were 28 males and 22 females. Among the 50 *H. pylori*-positive children, 30 successfully achieved eradication, and 20 did not. The mean plasma ghrelin levels of group 1 were 22.17 ± 1.73 ng/L and 26.59 ± 2.05 ng/L before and after the treatment, respectively, which was a significant increase (P = 0.001). However, the mean plasma ghrelin level of group 2 before and after the *H. pylori* treatment was 21.34 ± 2.40 ng/L and 22.24 ± 2.10 ng/L (P = 0.785). The plasma ghrelin levels increased substantially after treatment in group 1 but showed only minor changes in group 2. Similarly, the gastric ghrelin mRNA expression in group 1 before treatment was 2.84 ± 0.08. After treatment, the level was 3.11 ± 0.65, which was significantly different (P = 0.023). The gastric ghrelin mRNA expression in group 2 did not change significantly during the treatment (2.82 ± 0.44 *vs* 2.79 ± 0.31, P = 0.875). The plasma ghrelin and gastric ghrelin mRNA levels in group 1 increased substantially after the treatment but did not do so in group 2. In addition, the body mass index the two groups did not differ significantly 2 mo before and after the *H. pylori* treatment.

CONCLUSION: *H. pylori* eradication increases the plasma and tissue ghrelin levels in children with *H. pylori*-associated functional dyspepsia.

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Key words: *Helicobacter pylori*; Functional dyspepsia; Ghrelin; Eradication; Children

Peer reviewers: Tamara Vorobjova, Senior Researcher in Immunology, Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila, 19,

51014 Tartu, Estonia; Andrew S Day, MB, ChB, MD, FRACP, AGAF, Associate Professor, Department of Paediatrics, University of Otago, Christchurch, PO Box 4345, Christchurch 8140, New Zealand

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is an emerging global health problem. It is regarded as a major cause of chronic gastritis, duodenitis and peptic ulcers. *H. pylori* is generally acquired early in life[1,2]. The prevalence of *H. pylori* infection is higher in children from developing countries than in those from developed countries. *H. pylori* is the major pathogen of the gastrointestinal tract and may also be responsible for dyspeptic symptoms and reduced appetite that can lead to malnutrition and impaired growth[3,4].

The gastrointestinal hormone ghrelin is a recently discovered gut-brain peptide that regulates food intake in humans and has strong growth hormone-releasing activity[5,6]. Ghrelin is composed of 28 amino acids and has been confirmed to both stimulate the hypothalamic appetite center through neuropeptide YNPY/AGRP and to directly regulate gastrointestinal function and appetite[7,8]. In addition, ghrelin enhances gastrointestinal tract motility and accelerates gastric emptying, which explains its appetite-promoting effects[9,10]. Ghrelin is mainly synthesized and secreted by gastric endocrine cells. Thus, chronic persistent damage to the gastric mucosa, which occurs in *H. pylori* infection, may affect ghrelin production and lead to changes in food intake and body weight.

In adults, there are contradictory reports on the relationship between *H. pylori* infection and ghrelin. Some studies have demonstrated that *H. pylori* infection decreased ghrelin secretion[11,12], whereas other studies have reported that *H. pylori* infection has no effect on plasma ghrelin levels[13,14]. There have been only a few small studies evaluating the relationship between ghrelin and *H. pylori* infection in children. A direct relationship between *H. pylori* infection and gastric ghrelin production in children remains to be demonstrated.

Thus, we studied children with *H. pylori*-associated functional dyspepsia (FD) and evaluated their plasma and gastric ghrelin production before and after treating their *H. pylori* infection. FD is a syndrome characterized by chronic and recurrent gastroduodenal symptoms in the absence of any organic or metabolic disease. *H. pylori* infection has been suggested to play a role in the development of FD. FD in patients with *H. pylori* infection is defined as a different disease entity. We recruited FD subjects to minimize any confounding factors that might have affected the study.

MATERIALS AND METHODS

Subjects

This study examined children with FD and *H. pylori* infection that had been confirmed by positive bacterial culture results. The FD diagnosis was based on the Rome Ⅲ criteria[15]. Patients with histopathological examination showed normal mucosa. The children were divided into two groups, depending on the *H. pylori* treatment results: the *H. pylori* eradication group (group 1) and the *H. pylori* failed eradication group (group 2). The patients who had the following symptoms or diseases were excluded from the study: serious infections, heart, lung, kidney or other major organ disorders, use of drugs that may affect the *H. pylori* test results (antibiotics, H2 blockers, proton pump inhibitors and bismuth preparations) and use of corticosteroids. The patients who showed severe gastritis, ulcers or erosion by endoscopy were also excluded.

Endoscopy and biopsy samples

All of the participants underwent an initial upper gastrointestinal endoscopy. Six biopsy specimens were taken from the intact mucosa in the gastric antrum. Two of these specimens were used for bacterial cultures. Another two specimens were used for histological assessment. The remaining specimens were immediately snap frozen and stored in liquid nitrogen for ghrelin mRNA detection.

Radioimmunoassay for serum ghrelin levels

On the day of the endoscopy, blood samples were taken between 8:00 am and 10:00 am after an overnight fast, transferred into tubes containing EDTA and centrifuged. The plasma was then separated and stored at -80 ℃ until needed. The blood samples were taken before and after the *H. pylori* treatment. The plasma ghrelin concentrations were measured by radioimmunoassay.

Real-time reverse transcription polymerase chain reaction of ghrelin

Quantitative reverse transcription polymerase chain reaction was used to detect ghrelin mRNA. cDNA was generated using M-MLV reverse transcriptase (Sangon Biological Engineering Technology And Service Co., Shanghai, China), 1 μg of total RNA as a template and random hexamer priming. The resulting cDNA was amplified using an Exicycler. The real-time PCR analysis was conducted using SYBR® Premix Ex Taq™ and specific primers. The forward ghrelin primer was TGGAGGTCAAGCAGAAGGG, and the reverse primer was GCAGAAGCAAGCGAAAAGC. The mRNA levels were normalized using β-actin.

Bacterial culture and identification

Selective Columbia blood agar (containing 5 mg/L TMP, 10 mg/L vancomycin, 2500 U/L polymyxin and 2 mg/L amphotericin B) was used for the culture. The *H. pylori* identification was based on colony morphology, the catalase and urease tests, and Gram staining to determine the morphological and staining properties.

Table 1 The characteristics of the study population at baseline

	Group 1	Group 2
Number of children (male/female)	17/12	11/10
Age (yr)	6.15 ± 0.74	5.49 ± 0.23
BMI (kg/m^2)	14.7 ± 1.5	15.9 ± 1.7

Group 1: *Helicobacter pylori* (*H. pylori*) eradication group; Group 2: *H. pylori* failed eradication group. BMI: Body mass index.

H. pylori eradication

The *H. pylori*-infected children were treated with omeprazole (20 mg/d), amoxicillin (50 mg/kg per day), and clarithromycin (15 mg/kg per day) for two weeks. The children were not given any other medication after the triple therapy. The *H. pylori* infection status was evaluated using the 13C-urea breath test 2 mo after the end of the therapy.

Follow-up

The patients were followed up 2 mo after the end of therapy. Endoscopy and biopsy sampling were conducted, and plasma was collected again. We monitored serum ghrelin and gastric ghrelin mRNA and assessed the changes in their body mass indexs (BMIs).

Statistical analysis

All of the data were expressed as mean ± SE. The Student's *t* test was used to compare mean. $P < 0.05$ was considered to be significantly different.

RESULTS

From January 2009 to April 2011, we studied 50 children with *H. pylori*-associated FD. The characteristics of the children, including their mean age, gender and BMI, are shown in Table 1. The *H. pylori* therapy was effective in 30 children and failed in 20 children.

In group 1, the plasma ghrelin levels before and after the treatment were significantly different (22.17 ± 1.73 ng/L and 26.59 ± 2.05 ng/L, respectively, $P = 0.001$). However, the plasma ghrelin levels in group 2 before and after the treatment were similar (21.34 ± 2.40 ng/L and 22.24 ± 2.10 ng/L, respectively, $P = 0.785$). The plasma ghrelin levels in group 1 increased substantially after the therapy, but those in group 2 showed only minor changes.

We also compared gastric ghrelin mRNA expression in the two groups before and after the *H. pylori* treatment. Similar to the changes in the plasma ghrelin levels, the gastric ghrelin mRNA expression in group 1 was 2.84 ± 0.08 and 3.11 ± 0.65 ($P = 0.023$) before and after the treatment, respectively, which was significantly different. These values were not significantly different in group 2 (2.82 ± 0.44 and 2.79 ± 0.31, $P = 0.875$).

The plasma ghrelin levels and gastric ghrelin mRNA increased significantly in group 1 but showed inconsistent changes in group 2 (Figures 1 and 2). The plasma ghrelin levels paralleled the gastric ghrelin mRNA expression levels in the *H. pylori* eradication group. These results suggest that attenuating ghrelin production in the gastric mucosa accounts for the decrease in the plasma ghrelin concentrations.

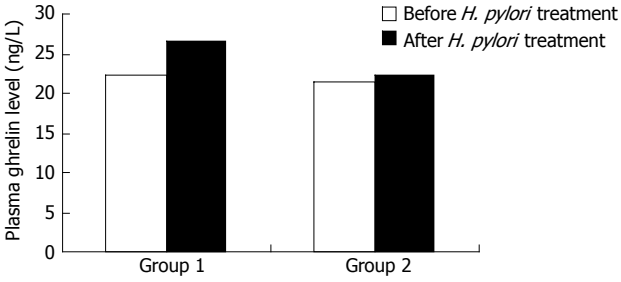


Figure 1 Plasma ghrelin levels before and after the *Helicobacter pylori* treatment in group 1 ($P = 0.001$) and group 2 ($P = 0.785$). Group 1: *Helicobacter pylori* (*H. pylori*) eradication group; Group 2: *H. pylori* failed eradication group.

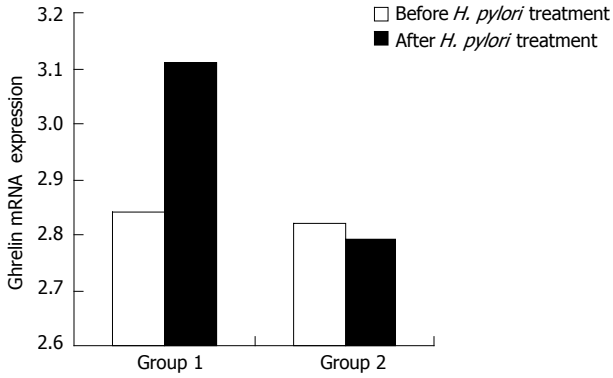


Figure 2 Ghrelin mRNA expression in the gastric mucosa before and after the *Helicobacter pylori* treatment in group 1 ($P = 0.023$) and group 2 ($P = 0.875$). Group 1: *Helicobacter pylori* (*H. pylori*) eradication group; Group 2: *H. pylori* failed eradication group.

The mean BMI in the *H. pylori* eradication group before and after the treatment was 14.7 ± 1.5 kg/m^2 and 15.6 ± 1.2 kg/m^2 ($P = 0.765$), respectively. The mean BMI in the *H. pylori* failed eradication group before and after the treatment was 15.9 ± 1.7 kg/m^2 and 15.5 ± 1.6 kg/m^2 ($P = 0.825$), respectively. Our study found that BMI did not change significantly before and after the *H. pylori* treatment in either group.

DISCUSSION

Our study found that the plasma ghrelin and gastric ghrelin mRNA levels increased significantly in those patients for whom the *H. pylori* treatment was successful and were not significantly different in those patients for whom the *H. pylori* treatment failed. These findings are consistent with those of other studies in which subjects underwent *H. pylori* treatment and had increased plasma ghrelin[16,17] and gastric ghrelin mRNA levels[18].

Inflammation of the gastric mucosa is the one of the important mechanisms of *H. pylori*-induced changes in ghrelin production. However, severe inflammation of the mucosal layers by other factors, such as ulcers and

atrophic gastritis, can also influence ghrelin production. Cummings *et al*[19] found that plasma ghrelin levels were low in a young woman with an evolving autoimmune gastric process. Thus, the change in ghrelin production caused by *H. pylori* infection might be confused with the severe inflammation caused by diseases such as ulcers and atrophic gastritis. Therefore, we recruited FD subjects without severe histopathological changes to minimize the confounding factors and found that ghrelin production increased after *H. pylori* eradication independent of inflammation. Furthermore, diseases such as ulcers, atrophic gastritis and cancer are seen much less frequently in children than in adults, and the host genetic and environmental factors (such as diet, alcohol, and smoking) that play an important role in ghrelin production are more uniform in children. Studies of children can clarify the relationship between *H. pylori* and ghrelin more directly than can studies of adults.

The reports on the influence of *H. pylori* eradication on ghrelin concentrations in children are contradictory. Pacifico *et al*[20] have reported that successful *H. pylori* treatment in children was associated with increased BMI and lean and fat mass but not with increased ghrelin levels, which were actually decreased. These results may suggest that changes in ghrelin levels after the *H. pylori* infection has been cured are epiphenomenal. By contrast, Konturek *et al*[21] found that eliminating *H. pylori* from the stomach using triple eradication therapy resulted in enhanced circulating plasma ghrelin in *H. pylori*-positive shepherds and their children. The discrepancies in the response to *H. pylori* treatment may be related to the strain of *H. pylori*. Patients with the type I strain, which expresses the cytotoxin-associated gene product and vacuolating cytotoxin A virulence factors, have lower circulating ghrelin levels than those with the less virulent type II strain, which does not express these virulence factors[22]. The features of *H. pylori* strains are known to differ between developed and developing countries, and *H. pylori* infection is also known to be associated with socio-economic conditions. The patients in the study by Konturek *et al*[21] were shepherds who may have the same *H. pylori* strain as our patients, and our results are similar to those reported by Konturek *et al*[21].

Ghrelin-producing cells have been found mainly in the oxyntic mucosa of the stomach. Decades of acquired *H. pylori* infection can lead to chronic gastritis and atrophy. Thus, some authors have demonstrated that ghrelin production was influenced by reductions in the number of oxyntic cells caused by atrophy. Cindoruk *et al*[14] have shown that eradicating *H. pylori* has no affect on the plasma ghrelin concentration in patients without atrophy. Osawa *et al*[11] have also suggested that impaired gastric ghrelin production in association with the atrophic gastritis caused by *H. pylori* infection accounted for the decreased plasma ghrelin concentration. There was no evidence of gastric atrophy or intestinal metaplasia in our study. The functional impairment of ghrelin-producing cells due to inflammation, perhaps mediated by cytokines, may account for reduced ghrelin production[23,24]. Recent studies have shown that H. pylori infection causes the release of many pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and IL-8, and influences decreases in ghrelin production[25]. Abiko *et al*[26] further confirmed the relationships among H. pylori infection, ghrelin secretion and cytokines. A comparison of the ghrelin level, appetite and body weight of *H. pylori*-infected *IL-1* gene knockout and wild-type mice showed no obvious changes in the knockout mice but significant reductions in the ghrelin level, appetite and body weight of the infected wild-type mice. These results indicated that *H. pylori* infection can affect ghrelin secretion *via* IL-1. Therefore, further investigations are required to understand the relationships among ghrelin, *H. pylori* infection and inflammatory cytokines in children.

Compared with previous studies of children, our study not only demonstrated that plasma ghrelin concentrations were influenced by *H. pylori* eradication but also focused on the gastric mucosa to better understand the effects of *H. pylori* infection on ghrelin expression changes. Increases in gastric ghrelin production may account for the higher concentrations of plasma ghrelin seen after *H. pylori* eradication.

A previous study has reported that BMI increased significantly 12 mo after *H. pylori* eradication[27]. Our study was designed to evaluate ghrelin changes at 2 mo after *H. pylori* treatment and was therefore too short to detect changes in BMI.

The changes in ghrelin production can be related to successfully eradicating the *H. pylori* infection. Ghrelin has also been suggested as a potential marker for *H. pylori* treatment success and may be an indicator of children who should be treated for *H. pylori* infection.

In conclusion, we demonstrated that plasma and gastric tissue ghrelin levels in children are increased after *H. pylori* eradication. *H. pylori* may influence ghrelin production. We also hypothesize that ghrelin may play an important role in the mechanism of *H. pylori*-associated FD in children. Future studies are needed to investigate this hypothesis.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection in children is higher in developing country. It not only is the major pathogen of the gastrointestinal tract but also may be responsible for dyspeptic symptoms and reduces appetite that might lead to malnutrition to impair growth. Ghrelin is primarily secreted from the stomach and has been implicated in the coordination of eating behavior and weight regulation. Thus, there exists the possibility that chronic persistent damage of the gastric mucosa, such as *H. pylori* infection, might affect ghrelin production and lead to changes in food intake and body weight. The direct relationship between *H. pylori* infection and gastric ghrelin production is needed to be demonstrated.

Research frontiers

Many studies assessing the relationship between *H. pylori* and Ghrelin in adults have been published but a comparable study in children is lacking. The important area in the study is selected children with *H. pylori*-associated dyspepsia as subjects and evaluates plasma and gastric ghrelin production before and after treating *H. pylori*.

Innovations and breakthroughs

Compared with previous studies in children, the authors' study not only demonstrates that plasma ghrelin concentrations are influenced after *H. pylori* eradication but also focused on the gastric musca to better understand the effects of *H. pylori* infection on the alteration of ghrelin expression. Increases in gastric ghrelin production may account for higher concentration of plasma ghrelin after *H. pylori* eradication. The authors' study also suggested that level of ghrelin can be considered as one of the maker of *H. pylori* successfully or not and can be one of the indication for children *H. pylori* infection therapy. Furthermore, this report provide novel insights for understanding the physiological function of ghrelin and its relation to associated disease.

Applications

The study results suggest that gherlin levels in plasma and gastric tissue in children are increased after *H. pylori* eradication and ghrelin may play an important role in the mechanism of *H. pylori*-associated dyspepsia in children.

Terminology

Ghrelin: Ghrelin is a gastrointestinal hormone and possesses strong growth hormone-releasing activity and is recently discovered gut-brain peptide that regulates food intake of human; Functional dyspepsia: Functional dyspepsia is a syndrome characterized by chronic and recurrent gastroduodenal symptoms in absence of any organic or metabolic disease.

Peer review

This is a novel study in which authors evaluate plasma and gastric ghrelin production before and after treating *H. pylori* in children with *H. pylori*-associated dyspepsia. The results suggest that ghrelin may play an important role in the mechanism of *H. pylori*-associated dyspepsia in children.

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BRIEF ARTICLE

Predictive value of neutrophil infiltration as a marker of *Helicobacter pylori* infection

Xiao-Qing Xu, Zhen-Hua Wang, Jing-Xian Liao, Xiao-Yu Chen, Wen-Zhong Liu, Shu-Dong Xiao, Hong Lu

Xiao-Qing Xu, Zhen-Hua Wang, Jing-Xian Liao, Xiao-Yu Chen, Wen-Zhong Liu, Shu-Dong Xiao, Hong Lu, Department of Gastroenterology, Shanghai Renji Hospital, Shanghai Institute of Digestive Disease, Shanghai Jiaotong University School of Medicine, 145 Middle Shandong Rd, Shanghai 200001, China

Author contributions: Xu XQ performed this work and wrote the manuscript; Wang ZH and Liao JX collected patient data; Chen XY performed the histological examination; Liu WZ and Xiao SD performed the endoscopy; Lu H designed the project, directed the study and revised the draft.

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Correspondence to: Hong Lu, MD, PhD, Department of Gastroenterology, Shanghai Renji Hospital, Shanghai Institute of Digestive Disease, Shanghai Jiaotong University School of Medicine, 145 Middle Shandong Rd, Shanghai 200001, China. honglu02@yahoo.com
Telephone: +86-21-63200874 Fax: +86-21-63266027

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Abstract

AIM: To evaluate the predictive value of neutrophil infiltration as a marker of *Helicobacter pylori* (*H. pylori*) infection.

METHODS: A total of 315 patients with dyspepsia symptoms who underwent upper gastrointestinal endoscopy were enrolled in this study. Biopsies were evaluated using the updated Sydney system. The medication history of all patients in the preceding 4 wk was recorded. The diagnosis of *H. pylori* infection was based on ^{13}C-urea breath test at least 4 wk after withdrawal of antisecretory drugs, antibiotics and related drugs. For the patients with subtotal gastrectomy, the diagnosis of *H. pylori* infection was based on anti-*H. pylori* immunoglobulin G (IgG) antibody. Serum anti-*H. pylori* IgG antibody was measured by enzyme-linked immunosorbent assays (Biohit, Finland).

RESULTS: The sensitivity, specificity, positive predictive value and negative predictive value of neutrophil infiltration in the diagnosis of *H. pylori* infection were 92.3%, 83.5%, 77.4% and 94.7%, respectively. Neutrophil infiltration of gastric mucosa in the histological analysis was strongly associated with *H. pylori* infection (77.4% *vs* 5.3% in the neutrophil infiltration negative group, P = 0.000). Moderate neutrophil infiltration was more frequent in *H. pylori* infection when compared to mild infiltration (81.8% and 75%, respectively), but did not reach statistical significance. For those patients with negative rapid urease test, *H. pylori* was detected in 73.2% of patients with positive neutrophil infiltration on histology. In patients with subtotal gastrectomy, the diagnostic accuracy of neutrophil infiltration in *H. pylori* infection was 50%.

CONCLUSION: Neutrophil infiltration is closely associated with *H. pylori* and may be recognized as a sign of this infection.

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Key words: *Helicobacter pylori*; Histology; Neutrophil; Sensitivity; Specificity

Peer reviewer: Dr. Tamara Vorobjova, Senior Researcher in Immunology, Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila, 19, 51014 Tartu, Estonia

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INTRODUCTION

Detection of *Helicobacter pylori* (*H. pylori*) infection is the first and the most important step in the treatment of *H. pylori*-associated diseases. Urease-based tests, including the rapid urease test (RUT) and $^{13}C/^{14}C$ urea breath test (UBT), are common in the clinical practice of gastrointestinal (GI) diseases. Because of the high prevalence of *H. pylori* infection and high incidence of gastric cancer in China, endoscopy is recommended as the first-line management option for uninvestigated dyspepsia patients[1]. Endoscopy examination can not only diagnose pathological changes in the stomach, but the RUT based on gastric biopsy can detect *H. pylo*ri infection and has the advantages of speed and accuracy. The urea breath test as a non-invasive choice is universally accepted as the "gold standard" for the diagnosis of *H. pylori* infection. However, as urease-based tests, the RUT and UBT have some limitations. Many medications including acid suppressors [e.g., proton pump inhibitors (PPI), H_2-receptor antagonists (H_2RA)], antibiotics and bismuth influence these tests and cause false negative results[2-7]. The detection of *H. pylori* in histology requires experienced pathologists and special staining. It becomes very difficult in cases with a low number of organisms and in cocci-shaped organisms[5,7,8].

In patients with subtotal gastrectomy due to gastric cancer, the examination and treatment of *H. pylo*ri are the key points in preventing the recurrence of gastric cancer. The detection of *H. pylo*ri in patients after gastrectomy is difficult due to low accuracy of the UBT in this situation[9,10].

Some studies have reported that *H. pylori* infection is suggested by the presence of active inflammation[11,12]. Neutrophil infiltration is a hallmark of active inflammation. It is unknown whether neutrophil infiltration may be a marker of *H. pylori* infection. In this survey, we enrolled: (1) randomly-selected patients; (2) patients with neutrophil infiltration but negative-RUT; and (3) patients with subtotal gastrectomy and neutrophil infiltration on routine endoscopy and biopsy histology to explore the predictive value of neutrophil infiltration in the diagnosis of *H. pylori* infection.

MATERIALS AND METHODS

Patients

Patients presenting with upper gastrointestinal symptoms and endoscopically proven functional dyspepsia and scarred ulcer were enrolled into the study from June 2010 to September 2011 in Renji Hospital, Shanghai. Patients with active peptic ulcer and gastric cancer were excluded. A total of three biopsy specimens from the antrum or gastric remnant were taken during each endoscopy. One biopsy was used for the RUT to detect *H. pylori* and the other two biopsies were analyzed histologically. Informed consent was obtained from all patients in the study. Medication in the preceding 4 wk and previous *H. pylori* treatment before endoscopy examination were recorded.

Study design

Study 1: Patients without previous gastric surgery or antibiotic therapy, bismuth, PPI or H_2RA in the preceding 4 wk were included in this study.

Study 2: Only patients with neutrophil infiltration on histological analysis, but negative RUT were candidates for entry into this study. Patients with previous gastric surgery were excluded.

Study 3: Ten subtotal gastrectomy patients with neutrophil infiltration on histological analysis were recruited into this study. Patients who had received previous *H. pylori* eradication therapy or antibiotic therapy, bismuth, PPI or H_2RA in the preceding 4 wk were excluded.

Histology

All specimens for histological analysis were fixed in 10% formalin. An experienced pathologist analyzed the histological features of each sample using hematoxylin and eosin staining and made the diagnosis according to the updated Sydney classification[13]. The histological parameters included histological activity (neutrophil infiltration), chronic inflammation, glandular atrophy and intestinal metaplasia. Active inflammation meant the presence of neutrophil polymorphs in a background of chronic inflammation. Each category (mild, moderate, and severe) resulted in a score on a scale of 0-3 (none, 0; mild, 1; moderate, 2; severe, 3). The pathologist blinded to any clinical information counted the two specimens and calculated the average.

Assessment of H. pylori infection

^{13}C-UBT: ^{13}C-UBTs were performed after endoscopy examination or 4 wk after withdrawal of antisecretory drugs, antibiotics and related drugs. *H. pylori* infection was considered positive when values were $^{13}CO2 \geqslant 4.0‰$ delta over baseline.

Serological antibody test: Blood samples (5 mL venous blood) were taken from each patient prior to endoscopy. These samples were stored before analysis and then centrifuged at -20 ℃. Serum anti-*H. pylori* immunoglobulin G (IgG) antibody was measured by enzyme-linked immunosorbent assays (Biohit, Finland) according to the manufacturer's instructions. The IgG cut-off value which determined samples as Hp-positive was 2.5-80 ng/L.

RESULTS

Characteristics of patients included

A total of 315 patients were recruited in this study, including 261 with functional dyspepsia, 44 with scarred peptic ulcer and 10 with gastric remnant after distal gastrectomy (Table 1). Of the 10 patients with distal gastrectomy, 4 had a history of complicated peptic ulcer

Table 1 Baseline demographic characteristics of subjects

	Study 1	Study 2	Study 3
No. of patients	137	168	10
Sex (male/female)	62/75	66/102	8/2
Age (average, range)	48.8 (21-79)	52.4 (21-81)	55.2 (50-77)
FD/scarred ulcer	123/14	138/30	N/A

FD: Functional dyspepsia; N/A: Not available.

Table 2 Results of study 1

Neutrophil infiltration in gastric mucosa	^{13}C- Urea breath test		
	Positive	Negative	Total
Positive	48	14	62
Negative	4	71	75
Total	52	85	137

Table 3 Results of study 3

No. of positive patients	Anti-HP IgG antibody	RUT	^{13}C-UBT
Mild neutrophil infiltration (n = 8)	4	1	0
Moderate neutrophil infiltration (n = 2)	1	1	1

Anti-HP IgG: Anti-*Helicobacter pylori* immunoglobulin G; RUT: Rapid urease test; UBT: Urea breath test.

disease and 6 had a history of gastric cancer.

Accuracy of neutrophil infiltration in the diagnosis of H. pylori infection

Study 1: The diagnosis of *H. pylori* infection was based on ^{13}C-UBT. Sixty-two cases were positive for neutrophil infiltration, including 40 with mild and 22 with moderate infiltration. Of these 62 patients, 14 had a negative ^{13}C-UBT and 48 had a positive ^{13}C-UBT. Of the 40 patients with mild neutrophil infiltration, 10 had a negative ^{13}C-UBT and 30 had a positive ^{13}C-UBT. Of the 22 patients with moderate neutrophil infiltration, 4 had a negative ^{13}C-UBT and 18 had a positive ^{13}C-UBT. Seventy-five cases were negative for neutrophil infiltration. Of these patients, 71 had a negative ^{13}C-UBT and 4 had a positive ^{13}C-UBT (Table 2). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of neutrophil infiltration in the diagnosis of *H. pylori* infection were 92.3%, 83.5%, 77.4% and 94.7%, respectively. Neutrophil infiltration was strongly associated with *H. pylori* infection (77.4% *vs* 5.3% in the neutrophil infiltration negative group, $P = 0.000$). Moderate neutrophil infiltration was more frequent in *H. pylori* infection when compared to mild infiltration (81.8% and 75%, respectively), however, this difference failed to reach statistical significance.

Study 2: In 168 patients with negative RUT and positive neutrophil infiltration on histological analysis, the diagnosis of *H. pylori* infection was based on positive ^{13}C-UBT. ^{13}C-UBT is the gold standard, and *H. pylori* was detected in 73.2% (123/168) of patients with negative RUT and positive neutrophil infiltration on histology. 27.6% (34/123) of patients had a history of medication use (antibiotic therapy, bismuth, PPI or H_2RA) in the preceding 4 wk. Of 122 patients with mild neutrophil infiltration, 87 had a positive ^{13}C-UBT and the diagnostic accuracy was 71.3%. Of 46 patients with moderate neutrophil infiltration, 36 had a positive ^{13}C-UBT and the diagnostic accuracy was 78.2%.

Study 3: For the patients with subtotal gastrectomy and positive neutrophil infiltration on histological analysis, the diagnosis of *H. pylori* infection was based on positive anti-*H. pylori* IgG antibody. Serum anti-*H. pylori* antibody is the gold standard, and *H. pylori* was detected in 50% (5/10) of patients including 8 with mild neutrophil infiltration and 2 with moderate neutrophil infiltration. The diagnostic accuracy was 50% (Table 3).

DISCUSSION

The accurate diagnosis of *H. pylori* infection is an important step in the treatment of *H. pylori*-associated diseases. However, the outcome of the most commonly used urease-based *H. pylori* tests (RUT and UBT) may be veiled by some drugs and gastrectomy resulting in false negative results. PPIs have a marked influence on gastric pH or *H. pylori* density, inducing bacteria to migrate to atypical sites, thus leading to false negative UBT results as well as false negative RUT results[3,14]. Antibiotics active against *H. pylori* can cause a temporary decrease in the bacterial load and hinder the detection of *H. pylori* infection[5]. Discontinuation of antisecretory drugs and antibiotics four weeks before detection of *H. pylori* is currently recommended[2]. However, it is impractical for some patients suffering from severe symptoms such as acid reflux and upper GI hemorrhage to discontinue drug treatment. Gastrectomy and reconstruction make the UBT unreliable[15]. *H. pylori* is also difficult to detect in cases of extensive intestinal metaplasia[16]. However, detection of *H. pylori* infection is very important for patients with early gastric cancer, peptic ulcer and chronic atrophic gastritis.

H. pylori is found within the mucous gel layer covering the gastric mucosa. It is characterized by infiltration of inflammatory cells into the gastric mucosa which are dependent on the production of proinflammatory cytokines, especially the potent neutrophil chemotactic and activating peptide interleukin (IL)-8[17]. IL-8 is produced by gastric epithelial cells and binds to the extracellular matrix producing a chemotactic gradient that directs neutrophil migration to the epithelial surface. The density of intraepithelial neutrophils is closely related to the extent of mucosal damage and to the intensity of *H. pylori* infection[18-21]. Some researchers have investigated the association between the activity of inflammation and

H. pylori infection and have shown that colonization of the human stomach by *H. pylori* can induce an inflammatory response that is characterized by dense mucosal infiltration by neutrophils[18,22,23]. According to the updated Sydney system, gastric specimens containing neutrophils indicate *H. pylori*-positive patients if a sufficient number of biopsies from both the antrum and corpus are taken[13]. A study by Warren *et al*[24] showed that neutrophils gradually disappeared after starting *H. pylori* treatment. Neutrophils were rapidly detected again if the treatment failed. Without treatment, the changes are life-long. However, there are no studies on the predictive value of neutrophil infiltration as a marker of *H. pylori* infection.

Serology testing is the preferred method for patients with bleeding ulcers and PPI consumption[8]. Fischbach *et al*[25] found that serum IgG, determined by ELISA, was correlated with the histological degree and the activity of gastritis. The antibody absorbance index was correlated with severe activity of gastritis in the antrum, but not with the severity of body gastritis[26]. Vorobjova *et al*[27] reported that seropositivity for CagA antibody was a sign of gastritis activity. In our study, we used anti-*H. pylori* IgG antibody as a standard to diagnose *H. pylori* infection in patients with subtotal gastrectomy. We found that the diagnostic accuracy was 50% for neutrophil infiltration as a marker of *H. pylori* infection in this situation.

Our study showed that the sensitivity, specificity, PPV and NPV of neutrophil infiltration in the diagnosis of *H. pylori* infection were 92.3%, 83.5%, 77.4% and 94.7%, respectively. Neutrophil infiltration was strongly associated with *H. pylori* infection. There was no significant difference between moderate and mild neutrophil infiltration in the diagnosis of *H. pylori* infection. For those with a negative RUT, *H. pylori* was detected in 73.2% of patients with positive neutrophil infiltration on histology.

In patients with subtotal gastrectomy due to a history of complicated peptic ulcer disease and gastric cancer, eradication of *H. pylori* infection can prevent ulcer relapse and metachronous gastric cancer in these patients. However, the UBT is not accurate in this situation and some patients are unable to discontinue medication. In our study, we used anti-*H. pylori* IgG antibody as a standard to diagnose *H. pylori* infection. We found that the diagnostic accuracy was 50% for neutrophil infiltration.

Detection of *H. pylori* infection is key step in the treatment of certain diseases. Medication and gastrectomy can result in false negative urease-based tests for *H. pylori*. Neutrophils are a sensitive indicator of the presence of *H. pylori*. In clinical practice, neutrophil infiltration in the gastric mucosa may provide the clinician with a clue for the diagnosis of *H. pylori* infection.

COMMENTS

Background

The accurate diagnosis of *Helicobacter pylori* (*H. pylori*) infection is the key role in the treatment of *H. pylori*-associated diseases. Urease-based tests, including the rapid urease test and the $^{13}C/^{14}C$ urea breath test, are common in the diagnosis of *H. pylori* infection. However, medications and surgery can cause false negative results in urease-based tests. Gastric mucosa with active inflammation on histology is highly suggestive of *H. pylori* infection and neutrophil infiltration is a hallmark of active inflammation.

Research frontiers

The predictive value of neutrophil infiltration as a marker of *H. pylori* infection was studied, which may provide clinicians with a clue for the diagnosis of *H. pylori* infection in clinical practice.

Innovations and breakthroughs

This study showed that neutrophil infiltration of gastric mucosa on histological analysis was strongly associated with *H. pylori* infection and suggested that neutrophil infiltration may be a sign of this infection.

Applications

The association between neutrophil infiltration and *H. pylori* infection, shown in the present study, may provide clinicians with a clue for the diagnosis of *H. pylori* infection and help them identify false negative results in urease-based tests.

Peer review

In this manuscript, the authors verified neutrophil infiltration of gastric mucosa in histology analysis was strongly associated with *H. pylori* infection and highlighted neutrophil infiltration can be considered as a sign of the infection, which may help the clinicians diagnose the infection of *H. pylori* more accurately.

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BRIEF ARTICLE

Quality of life after three kinds of esophagectomy for cancer

Jian Zeng, Jin-Shi Liu

Jian Zeng, Jin-Shi Liu, Department of Thoracic Tumor Surgery, Zhejiang Cancer Hospital, Hangzhou 310004, Zhejiang Province, China
Author contributions: Zeng J designed the research; Liu JS contributed analytic tools; Zeng J and Liu JS analyzed the data; and Zeng J wrote the paper.
Correspondence to: Dr. Jian Zeng, Attending Doctor, Department of Thoracic Tumor Surgery, Zhejiang Cancer Hospital, Hangzhou 310004, Zhejiang Province, China. jjhwwyqsw@163.com
Telephone: +86-571-86889013 Fax: +86-571-88145807
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Abstract

AIM: To evaluate quality of life (QOL) following Ivor Lewis, left transthoracic, and combined thoracoscopic/laparoscopic esophagectomy in patients with esophageal cancer.

METHODS: Ninety patients with esophageal cancer were assigned to Ivor Lewis (n = 30), combined thoracoscopic/laparoscopic (n = 30), and left transthoracic (n = 30) esophagectomy groups. The QOL-core 30 questionnaire and the supplemental QOL-esophageal module 18 questionnaire for patients with esophageal cancer, both developed by the European Organization for Research and Treatment of Cancer, were used to evaluate patients' QOL from 1 wk before to 24 wk after surgery.

RESULTS: A total of 324 questionnaires were collected from 90 patients; 36 postoperative questionnaires were not completed because patients could not be contacted for follow-up visits. QOL declined markedly in all patients at 1 wk postoperatively: preoperative and 1-wk postoperative global QOL scores in the Ivor Lewis, combined thoracoscopic/laparoscopic, and left transthoracic groups were 80.8 ± 9.3 *vs* 32.0 ± 16.1 (P < 0.001), 81.1 ± 9.0 *vs* 53.3 ± 11.5 (P < 0.001), and 83.6 ± 11.2 *vs* 46.4 ± 11.3 (P < 0.001), respectively. Thereafter, QOL recovered gradually in all patients. Patients who underwent Ivor Lewis esophagectomy showed the most pronounced decline in QOL; global scores were lower in this group than in the combined thoracoscopic/laparoscopic (P < 0.001) and left transthoracic (P < 0.001) groups at 1 wk postoperatively and was not restored to the preoperative level at 24 wk postoperatively. QOL declined least in patients undergoing combined thoracoscopic/laparoscopic esophagectomy, and most indices had recovered to preoperative levels at 24 wk postoperatively. In the Ivor Lewis and combined thoracoscopic/laparoscopic groups, pain and physical function scores were 78.9 ± 18.5 *vs* 57.8 ± 19.9 (P < 0.001) and 59.3 ± 16.1 *vs* 70.2 ± 19.2 (P = 0.02), respectively, at 1 wk postoperatively and 26.1 ± 28.6 *vs* 9.5 ± 15.6 (P = 0.007) and 88.4 ± 10.5 *vs* 95.8 ± 7.3 (P = 0.003), respectively, at 24 wk postoperatively. Scores in the left transthoracic esophagectomy group fell between those of the other two groups.

CONCLUSION: Compared with Ivor Lewis and left transthoracic esophagectomies, combined thoracoscopic/laparoscopic esophagectomy enables higher postoperative QOL, making it a preferable surgical approach for esophageal cancer.

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Key words: Esophageal cancer; Quality of life; Thoracoscope; Laparoscope; Esophagectomy

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INTRODUCTION

China has the highest incidence (13/0.1 million) of esophageal cancer worldwide. Surgery is often the main treatment modality for patients with resectable esophageal cancer because it is potentially curative in up to 40% of cases[1-3]. However, conventional radical surgeries incur a large amount of trauma and result in poor postoperative quality of life (QOL)[4-7]. Recently developed minimally invasive surgical treatments for esophageal cancer, such as laparoscopic surgery, may improve patients' postoperative QOL. We compared postoperative QOL in patients with esophageal cancer who underwent combined thoracoscopic/laparoscopic esophagectomy, conventional Ivor Lewis esophagectomy, or single-incision esophagectomy through the left thorax in our hospital in 2010.

MATERIALS AND METHODS

Participants were selected from a group of 90 patients (62 male, 28 female; aged 46-81 years) treated by the same surgical team under the direction of Jinshi Liu to avoid bias. Inclusion criteria were: patients with resectable esophageal cancer who underwent surgical resection in our hospital between January and December 2010. The postoperative pathological diagnosis was squamous cell carcinoma in all patients. Patients with diabetes mellitus, hypertension, or psychiatric disorders were excluded from the study sample. No participant had received radiotherapy or chemotherapy before surgery, and no tumor showed significant invasion or distant metastasis.

This clinical trial was registered on the Chinese Clinical Trial Registry website (No. ChiCTR-TRC-12001861). The study was approved by the Ethics Committee of our hospital.

The patients were prospectively assigned to one of three surgical treatment groups of 30 patients each based on their order of presentation: Ivor Lewis esophagectomy, combined thoracoscopic/laparoscopic esophagectomy, or left transthoracic esophagectomy (Table 1). We performed bilateral cervical lymphadenectomy in patients with middle thoracic cancer in the Ivor Lewis and combined thoracoscopic/laparoscopic esophagectomy groups because of the survival benefits associated with this procedure[8]. Cervical lymphadenectomy could not be performed in patients undergoing left transthoracic esophagectomy. During the execution of the study, two surgeries had to be converted to open operations because the huge tumor compressed the left principal bronchus, and another case was converted because of extensive pleural adhesion. To avoid bias, we excluded these three patients from the study sample and recruited additional participants to maintain a sample size of 30 patients per group.

Surgery

Combined thoracoscopic/laparoscopic esophagectomy: General anesthesia was administered to each patient with a double lumen tube. The patient was then placed in the left recumbent position, and a thoracoscope camera port was placed at the midaxillary line of the 7th intercostal space. The camera was inserted, and the location of the work port was adjusted under its guidance. In general, the posterior 7th intercostal axillary line and 5th intercostal infrascapular line were selected as main work ports, and the anterior 4th intercostal axillary line as a secondary work port. The intrathoracic esophagus was mobilized while taking care not to damage the bilateral recurrent laryngeal nerve and trachea, and the intrathoracic lymph node was dissected. The patient was then placed in a supine position and the stomach was mobilized under camera guidance. The main work port was generally located 1 cm below the costal arch at the midclavicular line on the right abdominal wall, and the secondary work port was located 40% along a line extending from the navel to the main work port. On the left side, the port was located 1 cm below the left costal arch at the anterior axillary line. After mobilization of the stomach and dissection of the celiac lymph node, a approximately 5-cm-long incision was made in the abdominal wall, the cardia and proximal stomach were lifted manually, and a linear cutter was used to construct a gastric tube. The gastric tube was then returned to the abdomen. Finally, anastomosis was performed at the left cervix with a stapler. If indicated, a bilateral cervical lymphadenectomy was performed.

Left transthoracic esophagectomy: Anesthesia was the same as for combined thoracoscopic/laparoscopic esophagectomy. The patient was placed in the right lateral position and the chest was entered at the 6th or 7th intercostal space according to the disease focus. The diaphragm was opened, the stomach was isolated, and a gastric tube was made. The intrathoracic esophagus was mobilized and local lymph nodes were cleared. Anastomosis was performed above or below the costal arch with a stapler.

Ivor Lewis esophagectomy: Anesthesia was the same as for combined thoracoscopic/laparoscopic esophagectomy. The patient was placed in a supine position and the abdomen was opened. The stomach was mobilized, a gastric tube was made, the celiac lymph node was dissected, and the abdomen was closed. The patient was then placed in a left recumbent position and the chest was entered through the 5th intercostal space. The intrathoracic esophagus was mobilized and local lymph nodes were dissected. Finally, anastomosis was performed at the cupula pleurae or left neck, according to the disease focus. The superior margin of the cancer was located endoscopically. Patients underwent neck anastomosis if the distance between the incisor and the superior margin of cancer was less than 25 cm, and otherwise underwent intrathoracic anastomosis. A bilateral cervical lymphadenectomy was performed in cases of left cervix anastomosis.

The posterior mediastinal route was used in all patients. All patients received thoracic epidural analgesia for 3 d postoperatively.

Table 1 Clinical data of 90 patients undergoing esophagectomy and intra- and postoperative variables in 90 esophagectomies

	Esophagectomy type		
	Left transthoracic	Ivor Lewis	Combined thoracoscopic/ laparoscopic
Clinical data			
Patients (*n*)	30	30	30
Sex			
Male	20	19	21
Female	10	11	9
Mean age (yr)	62.6 ± 8.4	58.4 ± 10.4	66.2 ± 9.8
Average tumor length (cm)	4.2	5.6	4.1
Intrathoracic anastomoses (*n*)	30	22	0
Left cervical anastomoses (*n*)	0	8	30
Clinical staging			
I	3	2	0
II	11	9	13
III	16	19	17
IV	0	0	0
Intra- and postoperative variables			
Operation time (min)	143 ± 23	287 ± 49	306 ± 67
Blood loss (mL)	210 ± 97	245 ± 46	276 ± 89
Postoperative complications (*n*)			
Anastomotic fistula	0	1	3
Chylothorax	0	1	0
Incision infection	0	1	0

Evaluation of quality of life

QOL was evaluated in all patients using the quality of life-core 30 questionnaire (QLQ-C30; ver. 3.0, in Chinese) and the supplemental quality of life-esophageal module 18 questionnaire (QLQ-ES18, in Chinese) for patients with esophageal cancer, both of which were developed by the European Organization for Research and Treatment of cancer (EORTC)[9,10]. For this evaluation, each patient was visited in person during hospitalization 1 wk before and 1 wk after surgery, and contacted by telephone at 12 and 24 wk postoperatively. Patients completed the self-administered questionnaires, with the assistance of their physicians and/or relatives in cases of reading or writing difficulty. These assistants explained questions to the patients and recorded responses. Because the patients fasted during the first postoperative week, food intake was not evaluated.

The QLQ-C30 (including the Chinese version) has been used in QOL studies of patients with all kinds of cancer, and has shown good reliability and validity[11,12]. This questionnaire includes a total of 30 items in five functional scales (physical, role, emotional, cognitive, and social), three general symptom scales (fatigue, nausea and vomiting, and pain), one global QOL scale, and six single-item measures of general symptoms or problems (dyspnea, insomnia, appetite loss, constipation, diarrhea, and financial difficulties). Responses to each item are structured on a four-point scale: "not at all" (scored as 1), "a little" (scored as 2), "quite a bit" (scored as 3), and "very much" (scored as 4). The global QOL scale ranges from "very poor" (scored as 1) to "excellent" (scored as 7). Higher functional and comprehensive QOL index scores indicate better functions and QOL, whereas higher symptomatic index scores indicate worse symptoms and lower QOL[13].

The QLQ-ES18 is a QLQ-C30 supplement that is applied specifically to patients with esophageal cancer. This questionnaire contains a total of 18 items assessing symptoms such as dysphagia, reflux, and coughing when swallowing. Responses to each item are structured on the same four-point scale used in the core questionnaire[10]. Blazeby *et al*[10] demonstrated that the QLQ-ES18 had good psychometric and clinical validity, and recommended its use in combination with the core questionnaire, the QLQ-C30, to assess QOL in patients with esophageal cancer. Numerous studies have shown that the combined use of the QLQ-C30 and QLQ-ES18 (including Chinese versions) reflects QOL objectively in patients with esophageal cancer[14-17].

Statistical analysis

Each questionnaire item score was converted linearly to a scale of 1-100 according to the EORTC scoring manual[10,12,18], and means and standard deviations were then calculated. Data were processed using the SPSS software (ver. 13.0; SPSS Inc., Chicago, IL, United States) and QOL indices were compared among groups using the independent-samples *t* test. General patient information was compared among groups using the χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

The three groups showed no significant difference in age, sex, average tumor length, or clinical stage.

The mean operation time for left transthoracic esophagectomy (143 ± 23 min) was shorter than those for combined thoracoscopic/laparoscopic esophagectomy (306 ± 67 min; $P < 0.01$) and Ivor Lewis esophagectomy (287 ± 49 min; $P < 0.01$). The amount of blood loss did not differ among the three groups. One case of anastomotic leakage, one incision infection, and one chylothorax occurred postoperatively in the Ivor Lewis group. In the combined thoracoscopic/laparoscopic esophagectomy group, three cases of anastomotic leakage occurred postoperatively. No complication was found in the left transthoracic esophagectomy group (Table 1). The incidence of postoperative complications did not differ among groups, and all patients recovered completely after surgery. All patients were alive at 24 wk after surgery. No cancer recurrence or metastasis was found in any patient during the 24-wk follow-up period.

In total, 324 questionnaires were collected from 90 patients; 36 postoperative questionnaires were not completed because the patients could not be contacted for follow-up visits. No significant difference in QOL indices was found among the three groups at 1 wk before surgery. Compared with preoperative scores, the QOL of all patients had declined significantly at 1 wk after surgery (Figures 1 and 2). Preoperative and 1-wk postoperative global QOL scores in the Ivor Lewis, combined thoracoscopic/laparoscopic, and left transthoracic

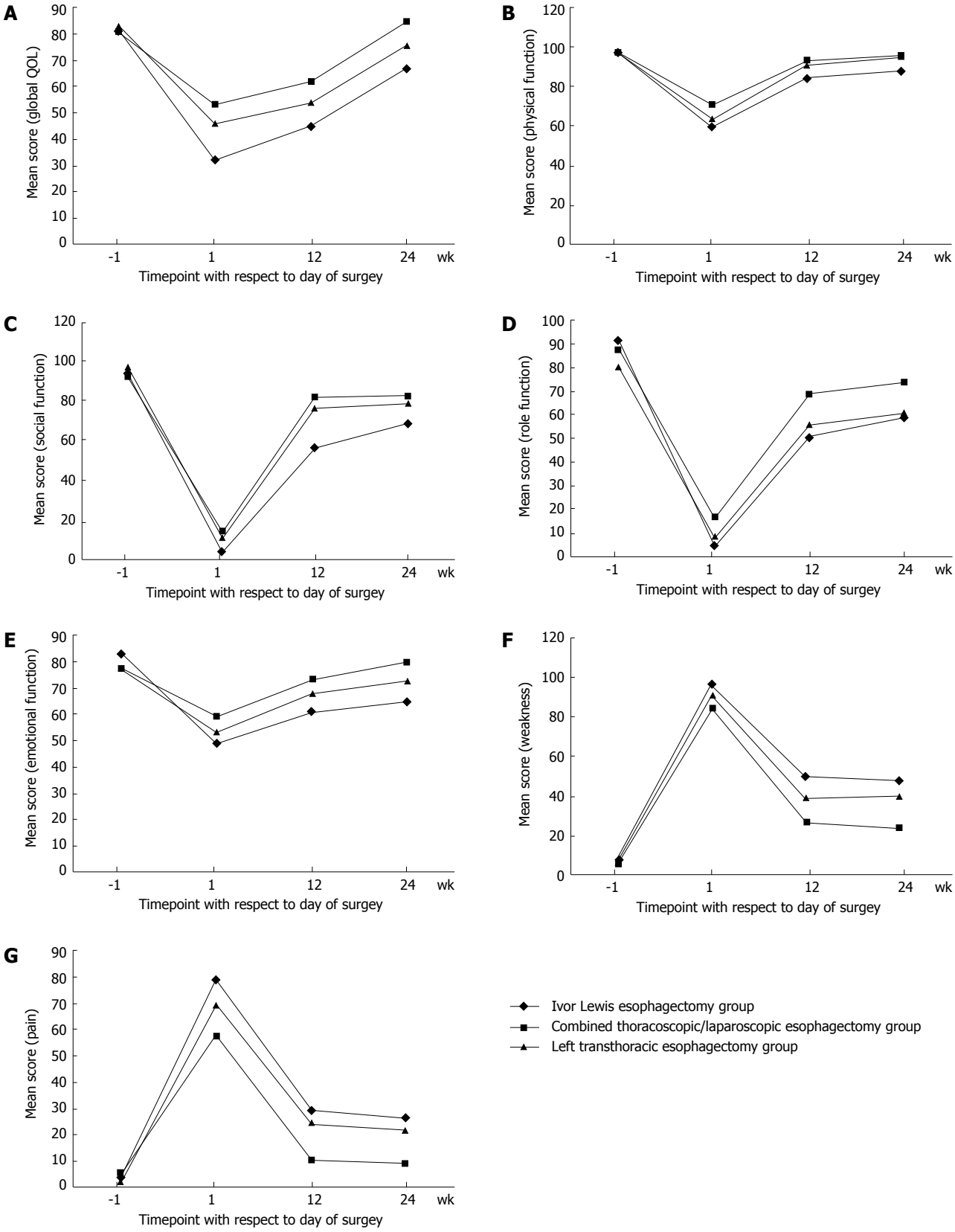


Figure 1 Quality of life, as determined by quality of life-core 30 questionnaire scores at 1 wk before and 1, 12 and 24 wk after surgery, in patients with esophageal cancer undergoing one of three esophagectomy procedures. A: Global scores; B: Physical function; C: Social function; D: Role function; E: Emotional function; F: Weakness; G: Pain. QOL: Quality of life.

groups were 80.8 ± 9.3 *vs* 32.0 ± 16.1, 81.1 ± 9.0 *vs* 53.3 ± 11.5, and 83.6 ± 11.2 *vs* 46.4 ± 11.3, respectively (all $P < 0.001$; Figure 1A). Most functional index scores decreased, with physical, emotional, social, and role scores

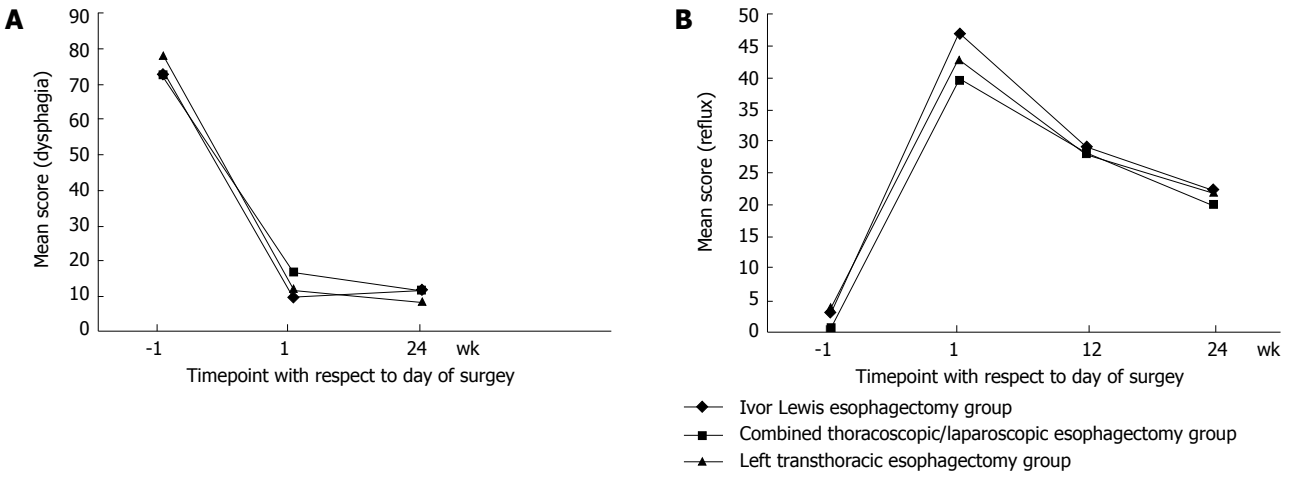


Figure 2 Severity of symptoms associated with esophageal cancer, as determined by quality of life-esophageal module 18 questionnaire scores at 1 wk before and 1, 12 and 24 wk after surgery, in patients with esophageal cancer undergoing one of three esophagectomy procedures. A: Dysphagia; B: Reflux.

showing the greatest declines (Figure 1B-E). The majority of symptomatic index scores, such as pain and weakness, increased (Figure 1F and G). QOL declined most in the Ivor Lewis group, which showed significantly lower physical and emotional function and significantly higher pain index scores compared with the combined thoracoscopic/laparoscopic esophagectomy group (all $P < 0.05$; Figure 1B, E and G). The degree of QOL decline in the left transthoracic esophagectomy group fell between those of the other two groups (Figures 1 and 2).

After surgery, QOL improved gradually in all three groups. All QOL indices were partially restored at 12 wk after surgery. Most functional indices approached preoperative levels in the combined thoracoscopic/laparoscopic esophagectomy group, but QOL status was not restored to preoperative levels in the left transthoracic esophagectomy and Ivor Lewis groups; the Ivor Lewis group showed the worst QOL status. Global QOL scores were higher in the combined thoracoscopic/laparoscopic group than in the other two groups at 1, 12 and 24 wk postoperatively (all $P < 0.05$; Figure 1A). Global QOL scores were higher in the left transthoracic group than in the Ivor Lewis group at 1 and 12 wk postoperatively (both $P < 0.05$), but showed no significant difference at 24 wk after surgery ($P = 0.053$). At 24 wk postoperatively, the incidence rates of pain in the combined thoracoscopic/laparoscopic esophagectomy, Ivor Lewis, and left transthoracic esophagectomy groups were 33.3%, 66.7% and 50%, respectively. All symptoms of dysphagia were alleviated in all groups (Figure 2).

Postoperative reflux occurred frequently after esophagectomy, and reflux scores showed no significant difference among groups during the 24-wk follow-up period. At 24 wk postoperatively, 66.7% of patients in the combined thoracoscopic/laparoscopic esophagectomy group complained of varying degrees of reflux, and rates were similar in the other two groups (Figure 2B).

DISCUSSION

The World Health Organization defined QOL as "an individual's perceptions of their position in life, in the context of the culture and value systems in which they live, and in relation to their goals, exceptions, standards and concerns"[19]. In the treatment of patients with esophageal cancer, more attention is usually given to the operation and postoperative survival rate than to QOL, even during long-term follow-up. However, patients often have many complaints about uncomfortable symptoms during this period; symptoms affecting postoperative QOL thus require further investigation and appropriate treatment. Today, within the context of the bio-psychosocial medical model, postoperative QOL should be included in the evaluation of treatment effectiveness. Unfortunately, not enough has been done in this respect. Thus, for patients with malignant neoplasms such as esophageal cancer, the improvement of QOL is especially important in the absence of a breakthrough in long-term survival rate improvement.

The surgical treatment of esophageal cancer has been a focus of modern medicine for more than 100 years. Given the deep location of the esophagus and the proximity of the intrathoracic esophagus to the heart, lungs, and other vital organs, esophagus substitutes are needed to restore the alimentary tract after the excision of the primary focus, and some doctors have advocated the "three-field esophagectomy" approach[20], such procedures are complex, difficult, and lengthy. Conventional open esophagectomy through the left thorax, a left thoracoabdominal incision or Ivor Lewis esophagectomy through the right thorax require the excision of the ribs and the use of a rib spreader, tearing part of the latissimus dorsi muscle or even severing the serratus anterior muscle. Thus, such surgery can result in major lesions that may not recover well after surgery and affect postoperative QOL. In this study, patients undergoing

left transthoracic or Ivor Lewis esophagectomy showed significant postoperative declines in QOL, and several index scores (i.e., physical, social, and emotional function indices; pain scores) had not recovered well at 24 wk postoperatively. However, patients undergoing left transthoracic esophagectomy showed better postoperative QOL than patients in the Ivor Lewis group, especially with respect to physical function index scores. This result was likely because left transthoracic esophagectomy produces fewer lesions than the Ivor Lewis procedure, the patient's position does not need to be changed during the operation, operation times are shorter, and the clearance is of smaller scope; left transthoracic esophagectomy thus inflicts less bodily harm, which benefits the restoration of postoperative QOL.

In the past 10 years, the updating of the theory of cancer therapy and improvements in technology and equipment, especially the introduction of new endoscopic imaging systems, have provided advantages for esophagectomy and lymphadenectomy such as the ability to perform these procedures under endoscopic guidance. With the accumulation of abundant experience in minimally invasive surgery for esophageal cancer, surgeons' skills, and thereby the clinical effects of these procedures, have improved[21-25]. Wang *et al*[26] compared short-term QOL in patients with esophageal cancer after subtotal esophagectomy *via* video-assisted thoracoscopic surgery or open surgery. They mobilized the stomach with dissection of the celiac lymph node by laparoscopy or laparotomy. However, QOL has not been compared previously in patients with esophageal cancer undergoing combined thoracoscopic/laparoscopic, Ivor Lewis, or left thorax esophagectomy.

Since 2010, when our hospital began to perform combined thoracoscopic/laparoscopic esophagectomy, we have performed this procedure in 50 patients with satisfying results. In this study, we found a smaller decline in postoperative QOL and a more rapid recovery in patients undergoing this procedure than in patients undergoing open esophagectomy, as indicated by symptomatic index and postoperative pain and weakness scores. Chronic pain complaints after thoracic surgery are clinically termed post-thoracotomy pain syndrome (PTPS). As an important QOL index, PTPS is a very common symptom that severely affects patients' satisfaction with life. PTPS represents a significant clinical problem in 25%-60% of patients, and intercostal nerve injury seems to be the most important pathogenic factor[27]. Because of the smaller thoracic wall cut, patients in our cohort experienced less pain after combined thoracoscopic/laparoscopic esophagectomy compared with open surgery. Because combined thoracoscopic/laparoscopic esophagectomy does not require the severing of the latissimus dorsi and serratus anterior muscles, most physical function index scores were restored to preoperative levels at 24 wk after surgery. The recovery of role, emotional, and social functions were also better in these patients than in the other two groups. Many patients scored lower in social and role functions due to postoperative surgical scars; thus, the smaller scars that remain after combined thoracoscopic/laparoscopic esophagectomy may help to restore patients' self-confidence, explaining why patients in this group scored higher than other groups on these two function indices.

A high incidence of postoperative reflux was observed in all of our patients during the 24-wk follow-up period; this might attributed to the elimination of the gastroesophageal sphincter mechanism preventing reflux, the persistence of gastric acid secretion, and the deterioration of esophageal clearance mechanisms[28,29]. In addition, gastric content may readily flow backward into the esophagus in the thoracic cavity due to negative pressure[30]. Although several anastomotic techniques have been used to construct a new antireflux barrier after esophagogastric junction resection[31-34], postoperative reflux after esophagectomy is a problem that warrants further attention and study.

Endoscopic esophagectomy has incomparable advantages given its minimally invasive nature in comparison with conventional surgery, but it also has some shortcomings. The procedure is less intuitive than an open operation, and thus has higher technical requirements for the operator. In cases of a huge tumor, obvious leftward displacement of the thoracic esophagus, or extensive pleural adhesion, endoscopic esophagectomy is very difficult to perform. As reported above, three cases in our series had to be converted to open operations for these reasons. However, with the continuous development of endoscopic equipment and improvement of surgical techniques, higher success rates of endoscopic esophagectomy are expected.

The currently leading viewpoint guiding the treatment of esophageal cancer is that the prognosis of this systemic disease depends mainly on biological activities and pathologic stage, whereas surgical treatment provides only local therapy. Thus, current surgical techniques of all types, including access through the left or right thorax and the use of one to three incisions, have little impact on patients' long-term survival[35,36]. Thus, if indications are precisely defined, appropriate cases are selected, and endoscopically guided operation skills are perfect, minimally invasive surgery should achieve long-term survival rates similar to those of open surgery[37,38]. Lazzarino *et al*[39] compared patients' survival status after minimally invasive or open esophagectomies performed in England between 1996 and 2007, and found better 1-year survival rates in patients undergoing minimally invasive procedures. On the basis of those findings, we performed minimally invasive surgery whenever possible for patients with esophageal cancer to improve their postoperative QOL. If camera-guided surgery is not available, an appropriate open surgical technique should be chosen according to the disease focus. For patients with distal esophageal cancer lacking evident mediastinal lymph node metastasis, we found that single-incision left transthoracic esophagectomy was much easier and pro-

duced fewer lesions. When the focal location is high or subcarinal lymph nodes or bilateral recurrent laryngeal nerves need to be cleared, the more thorough Ivor Lewis technique should be chosen.

This study had some limitations. The assignment of patients to the three groups was not strictly randomized, but was based on patients' order of presentation. Furthermore, endoscopic esophagectomy was a new operation carried out in our hospital during the study period, and surgical proficiency increased gradually. These factors may have introduced biases in the analysis. The 24-wk follow-up period was also relatively short and the sample size was small, preventing the comparison of long-term curative effects for these different esophageal cancer treatments. To evaluate long-term effects and survival rates, larger samples and further observation are needed.

COMMENTS

Background

Conventional radical operations for esophageal cancer incur a large amount of trauma and result in poor postoperative quality of life (QOL). The improvement of postoperative QOL in patients with esophageal cancer is particularly important in the absence of a breakthrough in long-term survival rate improvement.

Research frontiers

Recently developed minimally invasive surgical treatments for esophageal cancer, such as laparoscopic surgery, may improve postoperative QOL in patients with esophageal cancer.

Innovations and breakthroughs

In the past 10 years, the updating of the theory of cancer therapy and improvements in technology and equipment, especially the introduction of new endoscopic imaging systems, have provided advantages for esophagectomy and lymphadenectomy such as the ability to perform these procedures under endoscopic guidance. The authors compared postoperative QOL in patients after endoscopically guided esophagectomy or one of two other conventional open surgeries.

Applications

Compared with Ivor Lewis and left transthoracic esophagectomies, the study results suggested that combined thoracoscopic/laparoscopic esophagectomy resulted in higher postoperative QOL, making it a preferable surgical approach for esophageal cancer.

Terminology

QOL was defined by the World Health Organization as "an individual's perceptions of their position in life, in the context of the culture and value systems in which they live, and in relation to their goals, exceptions, standards and concerns."

Peer review

This is a good clinical study in which the authors compare patients' QOL following endoscopic esophagectomy or one of two other conventional open surgeries. The results are instructional and suggest that, in terms of postoperative QOL, combined thoracoscopic/laparoscopic esophagectomy is a preferable surgical approach for esophageal cancer.

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BRIEF ARTICLE

Health-related quality of life of 256 recipients after liver transplantation

Pei-Xian Chen, Lu-Nan Yan, Wen-Tao Wang

Pei-Xian Chen, Wen-Tao Wang, Department of Liver and Vascular Surgery, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China
Lu-Nan Yan, Department of Liver and Vascular Surgery, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China
Author contributions: Chen PX, Yan LN and Wang WT designed the study, performed the literature search and analyzed the data; Yan LN interpreted the results; and Chen PX wrote the paper.
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Correspondence to: Lu-Nan Yan, PhD, Department of Liver and Vascular Surgery, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China. yanlunan688@163.com
Telephone: +86-28-85422867 Fax: +86-28-85422469
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Abstract

AIM: To investigate health-related quality of life (HRQoL) and psychological outcomes in 256 adults who had undergone liver transplantation (LT).

METHODS: A stratified random sampling method was used in this follow-up multicenter study to select a representative sample of recipients undergoing either living donor liver transplantation (LDLT) or deceased donor liver transplantation (DDLT). HRQoL was measured by using the Chinese version of Medical Outcome Study Short Form-36 (SF-36), and psychological outcomes by using the beck anxiety inventory (BAI) and the self-rating depression scale (SDS). Clinical and demographic data were collected from the records of the Chinese Liver Transplant Registry and *via* questionnaires.

RESULTS: A total of 256 patients were sampled, including 66 (25.8%) receiving LDLT and 190 (74.2%) undergoing DDLT; 15 (5.9%) recipients had anxiety and four (1.6%) developed severe depression after the operation. Compared with LDLT recipients, DDLT patients had higher scores in general health (60.33 ± 16.97 *vs* 66.86 ± 18.42, $P = 0.012$), role-physical (63.64 ± 42.55 *vs* 74.47 ± 36.46, $P = 0.048$), role-emotional (61.11 ± 44.37 *vs* 78.95 ± 34.31, $P = 0.001$), social functioning (78.60 ± 22.76 *vs* 88.16 ± 21.85, $P = 0.003$), vitality (70.30 ± 15.76 *vs* 75.95 ± 16.40, $P = 0.016$), mental health (65.88 ± 12.94 *vs* 71.85 ± 15.45, $P = 0.005$), physical component summary scale (PCS, 60.07 ± 7.36 *vs* 62.58 ± 6.88, $P = 0.013$) and mental component summary scale (MCS, 52.65 ± 7.66 *vs* 55.95 ± 10.14, $P = 0.016$). Recipients > 45 years old at the time of transplant scored higher in vitality (77.33 ± 15.64 *vs* 72.52 ± 16.66, $P = 0.020$), mental health (73.64 ± 15.06 *vs* 68.00 ± 14.65, $P = 0.003$) and MCS (56.61 ± 10.00 *vs* 54.05 ± 9.30, $P = 0.037$) than those aged ⩽ 45 years. MCS was poorer in recipients with than in those without complications (52.92 ± 12.21 *vs* 56.06 ± 8.16, $P = 0.017$). Regarding MCS (55.10 ± 9.66 *vs* 50.0 ± 10.0, $P < 0.05$) and PCS (61.93 ± 7.08 *vs* 50.0 ± 10.0, $P < 0.05$), recipients scored better than the Sichuan general and had improved overall QoL compared to patients with chronic diseases. MCS and PCS significantly correlated with scores of the BAI ($P < 0.001$) and the SDS ($P < 0.001$).

CONCLUSION: Age > 45 years at time of transplant, DDLT, full-time working, no complications, anxiety and depression were possible factors influencing postoperative HRQoL in liver recipients.

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Key words: Liver transplantation; Living donor liver transplantation; Deceased donor liver transplantation; Psychology; Health-related quality of life

Peer reviewer: Dr. Seyed Mohsen Dehghani, Pediatric Gastroenterology, Shiraz University of Medical Sciences, Nemazee Hospital, Shiraz 7193711351, Iran

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INTRODUCTION

Liver transplantation (LT) has been an established therapy for various end-stage liver diseases for more than three decades. Aside from the role of ensuring the survival of individuals with liver disease, LT also offers patients the health they enjoyed prior to the disease, achieving a good balance between the functional efficacy of the graft and the patient's mental and physical integrity. Satisfactory overall long-term outcomes have been reported by many transplant centers around the world[1-3]. Saab *et al*[4] have reported that most patients were able to resume occupation early after liver transplantation. In addition, a Portuguese study showed that the majority of recipients could enjoy improved post-LT mental health[5]. However, Kousoulas *et al*[6] have published their results that patients who survived > 15 years after orthotopic LT demonstrated poor quality of life (QoL). It has also been suggested by de Kroon *et al*[7] that some patients exhibit physiological or psychological difficulties irrespective of favorable overall HRQoL.

Discordant conclusions with regard to post-LT overall QoL drawn from transplant centers have resulted in studies to identify potential influencing factors of health-related QoL (HRQoL). More than 10 years survival[8] and male sex[9] have been found to contribute to better post-LT QoL among recipients. Additionally, many studies have reported equivalent recipient results with living donor LT (LDLT) *vs* deceased donor LT (DDLT) in adult recipients. This was mainly indicated by similar short-term patient survival rates in both groups[10]. However, few studies have focused on the relationship of QoL, severity of depression, and incidence of anxiety. Thus, more careful analysis is warranted to ensure that a meaningful comparison is being made. The present study sought to evaluate carefully the HRQoL and psychological outcomes of adult recipients who underwent DDLT or LDLT between 2001 and December 2010 in Sichuan Province, China. We also aimed to establish the potential factors influencing QoL in our recipients after transplantation. In addition, comparisons of Short Form-36 (SF-36) scores were made between our sample and those of the Sichuan general population, patients with chronic illness, and 126 healthy controls.

MATERIALS AND METHODS

Follow-up model

To keep track of our recipients after transplantation and help them enjoy good physical and psychosocial recovery, our center developed its own follow-up model. At discharge, patients and their family members were lectured about what to pay attention to in daily life. It was also suggested that patients visit their doctors monthly in the first 6 mo, every 2 mo in the second 6 mo, and quarterly beyond 12 mo. In addition, patients were welcome to contact our transplant panel or their regular physicians by mail, telephone or fax if they had any transplant-related difficulties or it was inconvenient to come back. Regular contact was made to maintain follow-up in recipients. As soon as abnormalities were identified, patients underwent further examination or intervention or even readmission if necessary. Besides, an annual reunion was held to update our patients with information pertinent to medication and daily care, as well as to facilitate professional consultation. Simultaneously, sharing of feelings about surgery and postoperative life, experience of daily care, and maintaining good QoL were encouraged at the annual reunion. Therefore, no patient was lost to follow-up.

Study population and design

The study was designed as a follow-up multicenter study. A stratified random sampling technique was used to select a representative sample of recipients who underwent LT between 2001 and December 2010. Recipients aged ⩾ 18 years, with the ability to understand Chinese, who had undergone LT > 6 mo previously and had no severe complications or limited ability for self-expression were eligible for inclusion. A total of 300 recipients were selected; among whom 14 died at the time of investigation, 10 were < 18 years old, eight had received LT < 6 mo previously, five spoke their own ethnic language, six had severe medical complications or rejection, and one was diagnosed with schizophrenia. The resulting study sample included 256 recipients with 66 receiving LDLT and 190 receiving DDLT.

Clinical and demographic data were collected from records of the Chinese Liver Transplant Registry. Self-reported questionnaires (completed by interview or mail) were used to evaluate QoL and psychological outcomes.

Instruments

QoL was assessed using the validated Chinese version of the Medical Outcomes Study SF-36[11]. The SF-36 is a validated self-administered questionnaire used internationally to measure eight domains of health: physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health during the past 12 mo. The raw scores of each subscale were converted to scores that ranged from 0 to 100, with higher scores indicating higher levels of functioning or well-being. The level of HRQoL was assessed by comparing the mean value for the study sample with that for a representative sample of the general population of Sichuan Province[12]. The eight scale scores were aggregated into the norm-based physical and mental

component summary (PCS and MCS) scores that had a population mean of 50 and standard deviation of 10.

Beck anxiety inventory

This is a self-report assessment[13] in which each subscale consists of 21 items that are rated along a four-point Likert scale ("never", "sometimes", "often" or "always"). The participants were required to rate each item in the scale that best represented their current mood, especially during the past 2 wk. The established cut-point of $\geqslant 45$ corresponded to the likelihood of anxiety disorder.

Self-rating depression scale

The Zung *et al*[14] self-rating depression scale (SDS) was used in our study. It was a short self-administered survey to quantify the depressed status of a patient. There were 20 items on the scale that rated the four common characteristics of depression: the pervasive effect, the physiological equivalents, other disturbances, and psychomotor activities. There were 10 positively worded and 10 negatively worded questions. Each question was scored on a scale of 1-4 (based on these replies: "a little of the time", "some of the time", "good part of the time", "most of the time"). The ratio of the raw scores on the test > 80 was coined SDS indices, which are usually applied to assess the severity of the depressive symptoms of a patient. The SDS indices fell into four ranges: (1) < 0.50 normal; (2) 0.50-0.59 minimal to mild depression; (3) 0.60-0.69 moderate depression; and (4) $\geqslant$ 0.70 severe depression.

Ethical consideration

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and hence was approved by our Hospital Ethics Committee. All participants were asked to sign an informed consent form. There were no organs from prisoners and no subjects were prisoners.

Statistical analysis

Statistical analysis was performed using SPSS statistical software, version 17.0. Scores from recipients were respectively compared with that from the Sichuan general population; from patients with other chronic medical conditions, including type 2 diabetes mellitus[15], chronic hepatitis B[16,17]; and from 125 healthy persons[18]. Between-group differences in QoL were tested with independent-sample Student *t* test, analysis of variance, or nonparametric test as appropriate. Multiple comparisons for observed means were tested using the Student-Newman-Keuls procedure for which equal variances could be assumed and by the Games-Howell procedure when they could not be assumed. Pearson correlation analysis was used to analyze the relationships between the level of QoL and psychological outcomes. A probability value of $P < 0.05$ was taken to represent a significant difference.

Table 1 Recipient characteristics

Factors	*n* (%)
Age, yr (mean ± SD, 44.57 ± 9.97)	
⩽ 45	151 (59)
> 45	105 (41)
Sex	
Male	211 (82.4)
Female	45 (17.6)
Marital status	
Married	241 (94.1)
Unmarried	3 (1.2)
Divorced/widowed	12 (4.7)
Education attainment	
Elementary school	15 (5.9)
Middle school	108 (42.2)
University	133 (52)
Household income (RMB/mo)	
⩽ 1000	35 (13.7)
> 1000 to ⩽ 3000	153 (59.8)
> 3000	68 (26.6)
Etiology disease	
Hepatic cirrhosis	142 (55.5)
HCC	71 (27.7)
Severe hepatitis	30 (11.7)
Others	13 (5.1)
HCC/non-HCC	71 (27.7)/185 (72.3)
Complications (yes/no)	78 (30.5)/178 (69.5)
LT types (DDLT/LDLT)	190 (74.2)/66 (25.8)
Time since transplant	
⩽ 1 yr	38 (14.8)
> 1 yr, ⩽ 3 yr	54 (21.1)
> 3 yr, ⩽ 5 yr	66 (25.8)
> 5 yr	98 (38.3)
Employment after transplant	
Full-time	83 (32.4)
Part-time	75 (29.3)
None	98 (38.3)

HCC: Hepatocellular carcinoma; DDLT: Deceased donor liver transplantation; LDLT: Living donor liver transplantation.

RESULTS

Recipient characteristics

Informed consent for participation was obtained from all recipients and they all completed the validated questionnaires by interview or mail. The results from questionnaires completed by interview versus mail were not significantly different. The demographic and transplant-related characteristics of the study population are listed in Table 1. Most recipients were ⩽ 45 years at the time of transplantation (59.0%), were male (82.4%), had attained a higher education level (52.0%), and suffered from hepatic cirrhosis (55.5%). Many recipients remained or got married after transplantation (94.1%) and earned a salary between 1000 and 3000 RMB/mo (59.8%). Among our participants, 30.5% reported that they experienced early or late post-transplant complications including biliary events, endocrine disorders, physical or psychiatric problems, and 38.3% had received a liver transplant > 5 years ago, three of whom were > 10-year survivors. Roughly equal numbers of recipients

Table 2 Psychological outcomes after transplantation

BAI/SDS scores	Frequency	mean ± SD (%)
BAI		30.26 ± 7.44
< 45	241	94.1
⩾ 45	15	5.9
SDS		0.49 ± 0.11
< 0.5	114	44.5
⩾ 0.5, ⩽ 0.59	104	40.6
⩾ 0.6, ⩽ 0.69	34	13.3
⩾ 0.7	4	1.6

BAI: Beck anxiety inventory; SDS: Self-rating depression scale.

engaged in three post-transplant employment statuses (full-time, part-time and no employment). The majority of recipients (98.4%) insisted on regular follow-up with their regular physicians or community clinics. Approximately 74.2% of the recipients underwent DDLT and 25.8% received LDLT, all of whom were related to their donors.

Post-transplant QoL and psychological outcomes

Beck anxiety inventory (BAI): A mean score of 30.26 was obtained among 256 recipients, 15 (5.9%) of whom scored ⩾ 45 on the BAI, indicating the possibility of post-LT anxiety disorder (Table 2).

SDS: The SDS index reached an average level of 0.49 in our study sample**.** It was suggested that 142 out of 256 study participants exhibited depressive symptoms, with 104 mild, 34 moderate and four severe (Table 2).

Medical outcome study SF-36: In the general health (P = 0.012), role-physical (P = 0.048), role-emotional (P = 0.001), social-functioning (P = 0.003), vitality (P = 0.016), mental health (P = 0.005), PCS (P = 0.013) and MCS (P = 0.016) domains, recipients receiving DDLT scored higher than those undergoing LDLT. Patients aged ⩽ 45 years at the time of transplant scored lower than those > 45 years in vitality (P = 0.020), mental health (P = 0.003) and MCS (P = 0.037). MCS scores were lower in recipients with than without complications (P = 0.017). Recipients who resumed full-time employment scored higher in general health (P = 0.038), physical-functioning (P = 0.003), social-functioning (P = 0.018), bodily pain (P = 0.005), vitality (P = 0.038) and PCS (P = 0.001) than those who undertook part-time jobs or gave up working after transplantation (Table 3).

Scores from recipients were lower in physical-functioning (P < 0.001), role-physical (P = 0.001) but higher in vitality (P = 0.003), mental health (P = 0.001), PCS (P < 0.001) and MCS (P < 0.001) compared with that from Sichuan general population. Recipients had higher scores in general health (P < 0.001), social-functioning (P < 0.001), bodily pain (P < 0.001), vitality (P < 0.001), PCS (P < 0.001) and MCS (P < 0.001) than patients with chronic hepatitis B. In general health (P < 0.001), physical-functioning (P < 0.001), role-physical (P < 0.001), social-functioning (P < 0.001), bodily pain (P < 0.001), mental health (P = 0.001), patients after liver transplantation scored lower that the healthy controls. Compared with patients with type 2 diabetes mellitus, recipients had lower scores in physical-functioning (P = 0.001) and mental health (P < 0.001), as well as higher scores in role-physical (P < 0.001), role-emotional (P < 0.001), social-functioning (P < 0.001), bodily pain (P = 0.023) and vitality (P < 0.001) (Table 4).

The correlation coefficients between PCS and MCS scores of the SF-36 and the scores of BAI or SDS respectively are presented in Table 5. Both PCS and MCS were significantly correlated with the scores of BAI (P < 0.001) as well as the severity of depression based on the SDS (P < 0.001).

DISCUSSION

HRQoL, is a multifaceted construct of physiological and psychosocial aspects of well-being. Integrity of this construct represents an important measure of the success of a medical intervention beyond simple high survival rates, because HRQoL involves every single detail of a person's daily activity and social functioning. As a result, HRQoL of recipients undergoing LT has been increasingly valued. Earlier studies[19-21] have shown that DDLT contributes greatly to improved QoL. Emerging as a life-saving alternative because of organ shortage worldwide, LDLT has also enabled patients to achieve better QoL after than before surgery[22]. The present study aimed to investigate HRQoL in recipients undergoing either DDLT or LDLT.

Established evidence shows that a high level of post-LT anxiety is harmful in the long term to the well-being of the recipients[23], and depressive symptoms after LT are associated with an increased risk of long-term mortality[24]. Fortunately, the incidence of anxiety in our sample was low (5.9%) and those exhibiting severe depressive symptoms after surgery only accounted for a small proportion (4/256). Nevertheless, a high incidence of depressive symptoms (142/256) among recipients should not be underappreciated because of the low incidence of severe depression, because the frequency of depressive symptoms can impair QoL in liver recipients[25]. As a result, longitudinal studies and prolonged follow-up are required to disclose other possible factors that might undermine the psychological health of recipients.

In our study, patients receiving DDLT had significantly higher scores than those undergoing LDLT in the majority of domains on the SF-36. Smaller sample size in LDLT population could be one possible reason. Additionally, it was reported that total comprehensive cost[26], incidence of complications[27-30], rehospitalization rate[27], and HCC recurrence[31,32] were higher in LDLT than DDLT recipients. Higher blood level of tacrolimus in LDLT recipients was also pointed out, which indicated higher incidence of adverse effects[33]. These factors might all contribute to the poorer QoL in LDLT

Table 3 Recipient health-related quality of life

Factors SF-36 domains	Groups (mean ± SD)	Groups (mean ± SD)	Groups (mean ± SD)	*t*/*F* value	*P* value
LT types	DDLT	LDLT			
GH	66.86 ± 18.42	60.33 ± 16.97		*t* = 2.530	0.012
RP	74.47 ± 36.46	63.64 ± 42.55		*t* = 1.990	0.048
RE	78.95 ± 34.31	61.11 ± 44.37		*t* = 3.361	< 0.001
SF	88.16 ± 21.85	78.60 ± 22.76		*t* = 3.029	0.003
VT	75.95 ± 16.40	70.30 ± 15.76		*t* = 2.433	0.016
MH	71.85 ± 15.45	65.88 ± 12.94		*t* = 2.816	0.005
PCS	62.58 ± 6.88	60.07 ± 7.36		*t* = 2.507	0.013
MCS	55.95 ± 10.14	52.65 ± 7.66		*t* = 2.415	0.016
Age	≤ 45 yr	> 45 yr			
VT	72.52 ± 16.66	77.33 ± 15.64		*t* = -2.332	0.020
MH	68.00 ± 14.65	73.64 ± 15.06		*t* = -2.995	0.003
MCS	54.05 ± 9.30	56.61 ± 10.00		*t* = -2.097	0.037
Complications	Yes	No			
MCS	52.92 ± 12.21	56.06 ± 8.16		*t* = 2.412	0.017
Employment after transplant	Full-time	Part-time	None		
GH	69.31 ± 18.23	63.88 ± 18.51	62.67 ± 17.63[a]	*F* = 3.301	0.038
PF	88.67 ± 10.91	81.33 ± 17.66[a]	83.32 ± 13.64[a]	*F* = 5.809	0.003
SF	90.81 ± 20.01	85.67 ± 22.95	81.38 ± 23.28[a]	*F* = 4.069	0.018
BP	86.02 ± 13.38	78.91 ± 19.76[a]	78.76 ± 16.19[a]	*F* = 5.331	0.005
VT	78.25 ± 15.86	72.93 ± 17.63	72.50 ± 15.45[a]	*F* = 3.305	0.038
PCS	64.28 ± 6.20	60.87 ± 7.51[a]	61.93 ± 7.08[a]	*F* = 7.100	< 0.001

Only statistically significant data are displayed. [a]*P* = 0.05 *vs* group "Full-time". SF-36: Short Form-36; GH: General health; RP: Role-physical; RE: Role-emotional; SF: Social-functioning; VT: Vitality; MH: Mental health; PF: Physical functioning; BP: Bodily pain; LT: Liver transplantation; PCS: Physical component summary; MCS: Mental component summary.

Table 4 Health-related quality of life in liver recipients

SF-36 domain	LT (*n* = 256)	Sichuan general (*n* = 2249)	CHB (*n* = 150)	Healthy control (*n* = 126)	DM2 (*n* = 108)
General health	65.18 ± 18.25	67.30 ± 21.97	50.2 ± 24.0[a]	91.23 ± 11.02[a]	62.95 ± 23.87
Physical-functioning	84.47 ± 14.44	90.80 ± 15.07[a]	84.5 ± 15.6	76.36 ± 26.09[a]	87.38 ± 17.10[a]
Role-physical	71.68 ± 38.33	79.51 ± 34.70[a]	73.4 ± 27.4	82.79 ± 20.01[a]	62.73 ± 39.64[a]
Role-emotional	74.35 ± 37.89	76.45 ± 38.47	77.6 ± 23.8	69.46 ± 16.89	58.33 ± 43.74[a]
Social-functioning	85.69 ± 22.43	85.29 ± 18.06	78.6 ± 25.8[a]	74.44 ± 17.32[a]	77.78 ± 25.60[a]
Bodily pain	81.35 ± 16.17	82.41 ± 21.25	71.3 ± 26.0[a]	85.55 ± 18.02[a]	78.75 ± 25.48[a]
Vitality	74.39 ± 16.41	71.44 ± 15.81[a]	60.8 ± 23.6[a]	75.57 ± 25.71	66.16 ± 21.66[a]
Mental health	70.36 ± 15.05	73.52 ± 15.68[a]	72.0 ± 20.3	73.35 ± 21.26[a]	73.74 ± 21.08[a]
PCS	61.93 ± 7.08	50.00 ± 10.00[a]	42.8 ± 11.6[a]	-	-
MCS	55.10 ± 9.66	50.00 ± 10.00[a]	48.9 ± 12.3[a]	-	-

Health-related quality of life (HRQoL) in liver recipients respectively compared with Sichuan general population and patients with chronic hepatitis B (CHB) or type 2 diabetes mellitus (DM2) as well as 126 healthy controls. [a]*P*< 0.05 *vs* group "Liver transplantation (LT)". SF-36: Short Form-36; PCS: Physical component summary; MCS: Mental component summary.

Table 5 Correlation analysis between health-related quality of life and psychological outcomes

BAI/SDS scores	PCS (SF-36)		MCS (SF-36)	
	r	*P* value	*r*	*P* value
BAI	-0.397	0.001	-0.401	< 0.001
SDS	-0.271	0.001	-0.239	< 0.001

BAI: Beck anxiety inventory; SDS: Self-rating depression scale; SF-36: Short Form-36; PCS: Physical component summary; MCS: Mental component summary.

than DDLT recipients. However, these factors were not considered in the preliminary study design and hence further studies are warranted.

Employment is one measure of the ability to resume normal physical and socializing well-being. It has been shown that lack of disability income coverage prior to transplantation and high physical functioning[4], age and approaching retirement[12] are correlated with post-transplantation occupation. Among our 256 transplant recipients, age and worry about doing harm to the graft were the main reasons for part-time work status and unemployment instead of full-time work. Interestingly, recipients undertaking full-time work enjoyed better physical health than the other two according to the SF-36, regardless of higher scores for bodily pain. Early involvement in social activities and full-time work facilitate recipients normal physical and psychosocial well-being.

Other factors, including age and complications may

affect some QoL subscales. Age > 45 years at the time of transplant and no complications indicated better mental health in recipients. Duffy *et al*[1] have shown that age < 18 years at the time of transplantation was associated with improved long-term survival, and Jin *et al*[12] have concluded that post-transplant medical complications did not affect QoL or psychological outcomes.

In our study, sex, etiology of disease, time since transplant, marital status after transplant surprisingly turned out not to be associated with HRQoL in recipients after transplantation. Our results were in contrast with previous studies[1,12,34].

Regarding the SF-36, our sample had comparable scores in most domains to the Sichuan general population and scored significantly higher in both PCS and MCS. Our results were consistent with earlier studies[2,35,36]. A systematic review demonstrated that liver transplantation recipients experienced improved QoL with reference to the general population and compromised well-being when compared with healthy persons[36]. However, this is inconsistent with our findings. In our study, the LT recipients scored lower in general health, role-physical, bodily pain, mental health and higher in physical-functioning and social-functioning, as well as similarly in other domains when compared with 126 healthy controls.

LT patients are at high risk for cardiovascular events, impaired renal function and diabetes due to life-long immunosuppressive therapy[37]. Thus, there is a necessity to probe the QoL of LT recipients compared with matched controls. Overall HRQoL was significantly better in our LT recipients than patients with chronic hepatitis B or type 2 diabetes mellitus. The result was in accordance with the study of Duffy *et al*[1] about HRQoL of 20-year survivors after LT. Paradoxically, Elliott *et al*[36] have found that symptoms or functional abilities are not different between LT recipients and matched chronic liver disease controls. However, our findings were based on generic QoL instruments, which were not disease-targeted. Therefore, further studies with specific instruments are needed to explored factors contributing to the differences. With respect to the relationship between QoL and psychological outcomes, MCS and PCS scores significantly correlated with the scores of both BAI and SDS. This observation indicated that post-transplant anxiety and depression were potential factors influencing QoL, and our findings were inconsistent with earlier studies[12,38]. Attention should be paid to recipients who had difficulties in psychosocial adjustment after LT.

Generally, in our study recipients undergoing DDLT, age > 45 years, taking full-time work after transplantation, and absence of complications resulted in a favorable QoL. This result could be partly attributable to our follow-up model, which enabled early problem identification and prompt individual intervention as well as HRQoL maintenance and improvement of LT recipients. Telles-Correia *et al*[39] have pointed out that it is useful to design special modes of follow-up to improve patients' medical outcomes. van Ginneken *et al*[40] and Gross *et al*[41] have shared their successful experience of improving QoL of LT recipients by a rehabilitation program and a trial called Mindfulness-Based Stress Reduction.

Our study had several limitations. The findings were based on cross-sectional data, which are less informative than those of a longitudinal study. Nevertheless, our sample was relatively large. The sample size of LDLT patients was small, which could have led to biased results in group comparison. Recipients' anxiety for donors' health should be investigated in future studies because that might affect recipients' post-transplant mental health. Studies with specific instruments are also needed. Moreover, prospective studies are required to explore tailored intervention to help patients through physical and psychological discomfort as well as social disadvantages.

In conclusion, age > 45 years at the time of transplantation, DDLT, full-time working status and no complications after transplantation indicated better QoL in our recipients. Post-transplant anxiety and depression significantly correlated with MCS and PCS. A well-designed follow-up model plays an important role in improving post-LT survival and HRQoL of recipients.

COMMENTS

Background

Living donor liver transplantation (LDLT) is effective treatment for patients with end-stage liver disease, especially in Asian countries where the cadaveric graft supply is markedly limited. The graft and recipient survival rates are excellent and similar to those after deceased donor liver transplantation (DDLT). However, recipients suffer a wide range of physical, mental and psychological difficulties other than the risks inherent to the surgical process. Therefore, the health-related quality of life (HRQoL) of recipients after transplantation should be given priority.

Research frontiers

Currently, discordant conclusions as to HRQoL and psychological health of recipients after LT have been drawn based on varied instruments. Therefore, the study evaluated the HRQoL and psychological outcomes on DDLT or LDLT recipients and identified some underlying factors influencing their HRQoL, as well as delivering the perspectives.

Innovations and breakthroughs

This study is one of the few that have compared HRQoL in liver recipients and those suffering chronic diseases. Furthermore, the authors compared DDLT and LDLT. Additionally, the study was well constructed and was a comprehensive assessment of HRQoL and psychological well-being in recipients after LT.

Applications

The present study provides crucial preliminary findings on research into the recipients' QoL and psychological outcomes. The findings will play an important role in future clinical decision making.

Terminology

LDLT is a procedure in which a living healthy person voluntarily donates part of his/her liver to another without any commercial purposes. DDLT is a procedure in which the donated liver is procured from a cadaver. HRQoL is a multifaceted construct of physiological and psychosocial aspects of well-being.

Peer review

The study is well planned with HRQoL being compared between DDLT and LDLT and also patients with other chronic illnesses, healthy controls, as well as the general population. This was an interesting study in which the authors evaluated HRQoL and mental well being (anxiety and depression) in liver transplant recipients from Sichuan Province, China.

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BRIEF ARTICLE

Melatonin attenuates acute pancreatitis-associated lung injury in rats by modulating interleukin 22

Jia-Ping Huai, Xue-Cheng Sun, Meng-Jun Chen, Yin Jin, Xiao-Hua Ye, Jian-Sheng Wu, Zhi-Ming Huang

Jia-Ping Huai, Xue-Cheng Sun, Meng-Jun Chen, Yin Jin, Xiao-Hua Ye, Jian-Sheng Wu, Zhi-Ming Huang, Department of Gastroenterology, First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China
Author contributions: Wu JS and Huang ZM designed the research; Huai JP, Chen MJ and Ye XH performed the research; Sun XC and Jin Y provided analytic tools; and Huai JP wrote the paper.
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Correspondence to: Jian-Sheng Wu, Professor of Medicine, Department of Gastroenterology, First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China. wzwujs@163.com
Telephone: +86-577-88068257 Fax: +86-577-88068257
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Abstract

AIM: To investigate whether therapeutic treatment with melatonin could protect rats against acute pancreatitis and its associated lung injury.

METHODS: Seventy-two male Sprague-Dawley rats were randomly divided into three groups: the sham operation (SO), severe acute pancreatitis (SAP), and melatonin treatment (MT) groups. Acute pancreatitis was induced by infusion of 1 mL/kg of sodium taurocholate (4% solution) into the biliopancreatic duct. Melatonin (50 mg/kg) was administered 30 min before pancreatitis was induced, and the severity of pancreatic and pulmonary injuries was evaluated 1, 4 and 8 h after induction. Serum samples were collected to measure amylase activities, and lung tissues were removed to measure levels of mRNAs encoding interleukin 22 (IL-22) and T helper cell 22 (Th22), as well as levels of IL-22.

RESULTS: At each time point, levels of mRNAs encoding IL-22 and Th22 were significantly higher ($P < 0.001$) in the MT group than in the SAP group (0.526 ± 0.143 *vs* 0.156 ± 0.027, respectively, here and throughout, after 1 h; 0.489 ± 0.150 *vs* 0.113 ± 0.014 after 4 h; 0.524 ± 0.168 *vs* 0.069 ± 0.013 after 8 h, 0.378 ± 0.134 *vs* 0.122 ± 0.015 after 1 h; 0.205 ± 0.041 *vs* 0.076 ± 0.019 after 4 h; 0.302 ± 0.108 *vs* 0.045 ± 0.013 after 8 h, respectively) and significantly lower ($P < 0.001$) in the SAP group than in the SO group (0.156 ± 0.027 *vs* 1.000 ± 0.010 after 1 h; 0.113 ± 0.014 *vs* 1.041 ± 0.235 after 4 h; 0.069 ± 0.013 *vs* 1.110 ± 0.213 after 8 h, 0.122 ± 0.015 *vs* 1.000 ± 0.188 after 1 h; 0.076 ± 0.019 *vs* 0.899 ± 0.125 after 4 h; 0.045 ± 0.013 *vs* 0.991 ± 0.222 after 8 h, respectively). The mean pathological scores for pancreatic tissues in the MT group were significantly higher ($P < 0.01$) than those for samples in the SO group (1.088 ± 0.187 *vs* 0.488 ± 0.183 after 1 h; 2.450 ± 0.212 *vs* 0.469 ± 0.242 after 4 h; 4.994 ± 0.184 *vs* 0.513 ± 0.210 after 8 h), but were significantly lower ($P < 0.01$) than those for samples in the SAP group at each time point (1.088 ± 0.187 *vs* 1.969 ± 0.290 after 1 h; 2.450 ± 0.212 *vs* 3.344 ± 0.386 after 4 h; 4.994 ± 0.184 *vs* 6.981 ± 0.301 after 8 h). The severity of SAP increased significantly ($P < 0.01$) over time in the SAP group (1.088 ± 0.187 *vs* 2.450 ± 0.212 between 1 h and 4 h after inducing pancreatitis; and 2.450 ± 0.212 *vs* 4.994 ± 0.184 between 4 and 8 h after inducing pancreatitis).

CONCLUSION: Melatonin protects rats against acute pancreatitis-associated lung injury, probably through the upregulation of IL-22 and Th22, which increases the innate immunity of tissue cells and enhances their regeneration.

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Key words: Interleukin 22; Melatonin; Pancreatitis-associated lung injury; Severe acute pancreatitis; T helper 22 cell

Peer reviewers: Maha Maher Shehata, Professor, Internal

Medicine, Mansoura University, Medical Specialized Hospital, 35516 Mansoura, Egypt; Hongjoo Kim, Professor, Sungkyunkwan University Kangbuk Samsung Hospital, 108, Pyung-Dong, Jongro-Ku, Seoul 110-746, South Korea

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INTRODUCTION

Severe acute pancreatitis (SAP) is an inflammatory disease characterized by tissue edema, acinar necrosis, hemorrhage, and fat necrosis in the pancreas. Subsequent entry of proteolytic enzymes and vasoactive mediators into the systemic circulation always leads to multi-organ complications[1]. Pulmonary dysfunction is the most prominent manifestation of extra-abdominal organ dysfunction in pancreatitis, and its severity ranges from mild oxygenation abnormalities to severe acute respiratory distress syndrome (ARDS)[2]. Respiratory failure is associated with approximately 60% of the deaths that occur within the first week of pancreatitis[3,4]. Thus, the success of efforts to identify new therapeutic strategies for SAP will likely depend largely on understanding the molecular pathogenesis of damage to both the pancreas and the lungs.

Interleukin 22 (IL-22) is a member of the IL10 family of cytokines. It is an important effector of activated IL-22-producing T helper cells 22 (Th22), Th1 cells, and Th17 cells, as well as cytotoxic T-cell subsets, γδT cells, natural killer (NK), and NKT cells[5-7]. Activated T cells and NK cells are major sources of IL-22, and the highest levels of IL-22 expression have been detected in CD4+ memory cells. In contrast, IL-22 is not produced by either resting or activated monocytes, or B cells[8]. Like all other members of the IL10 family, IL-22 exerts its biological effects *via* heterodimeric transmembrane receptor complexes that each comprise of a type-1 and a type-2 receptor chain. These receptor chains belong to the cytokine receptor family class 2[5,9].

Since its discovery in 2000, several research groups have studied the unique biological activities of IL-22 and its significance in diseases. IL-22 protects tissues from damage, enhances tissue repair, maintains tissue integrity, increases innate immunity, and promotes anti-microbial defenses[5,10]. Therapy involving IL-22 improves liver regeneration after 70% hepatectomy[11] and also ameliorates high-fat diet-induced liver lipogenesis and hepatic steatosis[12]. Similarly, therapy with IL-22 counteracts the destructive nature of inflammatory bowel disease and ulcerative colitis[13] and prevents lung inflammation and fibrosis[14].

The recently discovered Th22 subset of T cells, which differs from Th17 and other known T-cell subsets based on its unique function[15], produces cytokines such as IL-22, IL26, and IL13. Th22 cells express several fibroblast growth factors, are associated with epidermal repair responses, and synergize with tumor necrosis factor α to induce a characteristic Th22 signature in keratinocytes[15]. Moreover, Th22 cells may regulate epidermal responses in inflammatory skin diseases and could be involved in unknown pathways that control tissue immunity and remodeling[15].

Although most melatonin is produced by the pineal gland, it can also be secreted by extrapineal organs and tissues such as the gastrointestinal tract, the retina and lens, skin, immune and hematopoietic cells, and some reproductive organs[16,17]. Several studies have demonstrated the beneficial effects of melatonin on SAP. As early as 1999, melatonin was reported to attenuate pancreatic edema and lipid peroxidation in caerulein-induced SAP[18]. Subsequent studies confirmed that pretreatment with melatonin significantly decreases all investigated inflammatory parameters associated with SAP[18]. Moreover, melatonin exerts an anti-inflammatory effect by inhibiting nuclear factor kappa B, a transcription factor with a central role in the development of inflammatory diseases. The present study was undertaken to examine the role of IL-22 in the pathogenesis of AP-induced lung damage, as well as the capacity of melatonin to protect against AP-associated lung injury.

MATERIALS AND METHODS

Animals

A total of 72 clean-grade healthy adult male Sprague-Dawley (SD) rats, each weighing 200-250 g, were obtained from the Experimental Animal Center of Wenzhou Medical College, Wenzhou, China. All animals were fed standard rat chow and had unlimited access to water under conditions that provided a constant room temperature of 25 ℃ and a 12-h/12-h day/night cycle. All animals were acclimated for at least a week before experimentation. Animals were deprived of rat chow for 12 h before experimentation but were allowed unlimited access to water throughout the experimental period. All procedures were performed in accordance with the guidelines for animal experiments of Wenzhou Medical College, Wenzhou, China.

Animal groups and procedures

SD rats were anesthetized by intraperitoneal (i.p.) injection of 10% chloraldurate (2 mL/kg; Solarbio, Beijing, China) and were randomly assigned to a sham operation group (SO group; n = 24), a SAP group (n = 24), or a melatonin treatment group (MT group; n = 24). For each animal in the SAP group, a laparotomy was performed through a midline incision, and 1 mL/kg of 4% sodium taurocholate (Sigma, St. Louis, United States) was retrogradely injected into the biliopancreatic duct through the papilla using a segmental epidural catheter *via* a microinjection pump at a rate of 0.2 mL/min. A microclip was placed in the hepatic portion of the biliopancreatic duct to avoid reflux before injection of sodium taurocholate.

Rats in the SO group were subjected to the same surgical procedure but without infusion of 4% sodium taurocholate. In the MT group, melatonin (50 mg/kg body weight; Sigma) was administered i.p. 30 min before the injection of taurocholate. After each operation, the abdomen of each rat was closed in two layers. All surgical procedures were performed using sterile techniques.

The rats were killed by exsanguination at defined time points (1, 4 and 8 h; $n = 8$ for each time point) after the surgical procedure. Postcava puncture was used to obtain 3 mL blood from each animal to assay levels of serum amylase. The concentration of serum amylase (U/L) was assayed using a fully automatic biochemical analyzer (Hitachi, Tokyo, Japan). Lung tissues were harvested immediately before death, and tissue samples were fixed in 4% paraformaldehyde for immunohistochemical analysis. Other tissues were removed and stored in liquid nitrogen for the determination of IL-22 and Th22 mRNA levels using real-time reverse-transcription polymerase chain reaction and IL-22 protein levels using enzyme-linked immunosorbent assay (ELISA).

Histological analysis and pathological scores of pancreatic tissues

Samples of lung and pancreatic tissues were fixed in 40 g/L formaldehyde, embedded with paraffin wax, sectioned (4 μm thick), stained with hematoxylin and eosin (H and E), and observed using light microscopy. Tissue alteration was assessed (20 fields/section) by an experienced histologist who was blinded to the experimental protocol. Ten randomly selected visual fields in each pathological section were observed (H and E staining; × 400) using a high-powered microscope (CX31, Tokyo, Japan) and scored by two experienced pathologists using established standards[19], respectively. The maximum possible score for each visual field was 12, and the pathological score for each section was determined by calculating the mean of the scores from the two pathologists for the 10 visual fields.

Real-time, fluorescence-based quantitative polymerase chain reaction

Total RNA was extracted from lung tissues using TRIzol reagent (Invitrogen, Carlsbad, United States), and cDNA was synthesized using the first strand cDNA synthesis kit (MBI Fermentas, Burlington, Canada). mRNAs encoding IL-22 and Th22 were detected using the PCR mix kit (Daweike, Shanghai, China), and real-time quantitative PCR was performed using the ABI 7500 Sequence Detection System (Applied Biosystems Inc, Carlsbad, United States). For PCR, we used the following primers for IL-22 (forward: 5'-GCCAGCTGCCTGCTTCTCGT-3', reverse: 5'-CTGGCCTCCTTGGCCAGCAT-3'), Th22 (forward: 5'-GGGCTTCCAGGGTGCTTCGC-3', reverse: 5'-CCTCAGTTCACCGAGAACCCCA-3'), and β-actin (forward: 5'-GCGTCCACCCGCGAGTACAA-3', reverse: 5'-CGACGACGAGCGCAGCGATA-3'; Generay, Shanghai, China). The cDNA was denatured at 95 ℃ for 5 min and amplified over the course of 40 cycles, each involving 95 ℃ (15 s), 60 ℃ (45 s), and 72 ℃ (60 s), with a final extension at 72 ℃ (5 min). The samples were tested in triplicate, and the expression of IL-22 and Th22 mRNA was calculated using the $2^{-\Delta\Delta CT}$ method.

Sandwich enzyme-linked immunosorbent assays

Concentrations of IL-22 were measured using commercially available sandwich ELISA kits (all from R and D Systems, Rapidbio, United States). IL-22 levels were assayed using microtiter wells coated with purified rat IL-22 and a solid-phase IL-22-specific antibody. The horseradish peroxidase (HRP)-labeled secondary antibody was detected with the tetramethylbenzidine substrate solution, which creates a blue reaction product. The HRP enzyme-catalyzed reaction was terminated by the addition of a sulfuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of IL-22 in the samples was then determined by comparing the $A_{450\ nm}$ of the samples to a standard curve.

Statistical analysis

Statistical analysis were carried out using the SPSS software (ver. 15.0; SPSS Inc., Chicago, IL, United States). All data are expressed as the mean ± SD. One-way analysis of variance were used to investigate differences among the SO, SAP, and MT groups at each time point, and post hoc comparisons were performed between samples in the SO and SAP groups to check for statistical significance. Differences were considered significant at $P < 0.05$.

RESULTS

Histopathological examination of lung tissues and pathological scores of pancreatic tissues after SAP induction

The appearance of lung tissue samples collected 1 h (Figure 1A), 4 h (Figure 1B), and 8 h (Figure 1C) after the induction of SAP was much more severely affected than the appearance of tissue from animals in the SO group (Figure 1D), which tended to remain morphologically normal even 8 h after surgery. Microscopic examination of lung tissue from animals in the SAP group provided extremely clear evidence of edema, hemorrhage complicated by microthrombosis, cell degeneration, and inflammation (Figure 1A-C). The extent of damage observed in the lungs of SAP animals was largely alleviated in lung tissues of animals in the MT group (Figure 1E-G).

Pancreatic tissue samples were also collected and analyzed from each group of animals. At each time point, the mean pathological score for pancreatic tissues in the MT group was significantly higher ($P < 0.01$) than for samples in the SO group, but significantly lower ($P < 0.01$) than for samples in the SAP group at each time point. The severity of SAP increased significantly over time in

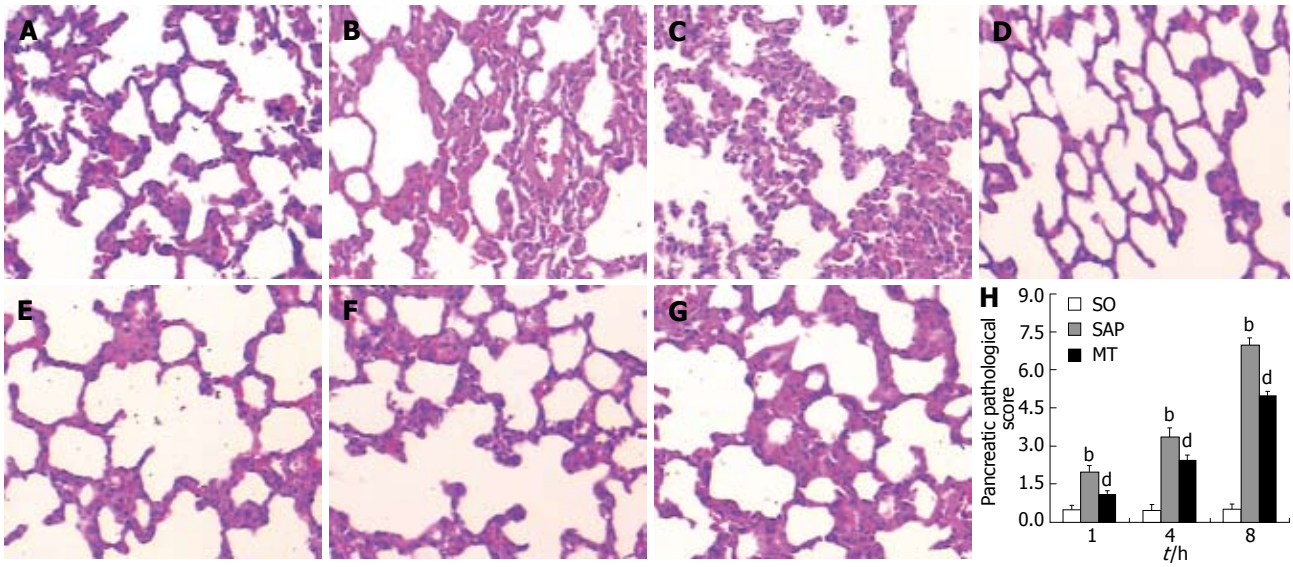


Figure 1 Histology of pulmonary tissues and pathological scores of pancreatic tissues from rats in the sham operation, severe acute pancreatitis, and melatonin treatment groups. A-C: Histological changes of pulmonary tissues in the SAP group (A) 1 h, (B) 4 h, and (C) 8 h after experimental induction of SAP; D: Histological changes of pulmonary tissues in the SO group 8 h after mock induction of SAP; E-G: Histological changes of pulmonary tissues in the MT group (E) 1 h, (F) 4 h, and (G) 8 h after experimental induction of SAP (hematoxylin and eosin stain; ×200). H: Pathological scores of pancreatic tissues (n = 8). $^{b}P < 0.01$ *vs* the MT group; $^{d}P < 0.01$ *vs* the SO and MT groups. SO: Sham operation; SAP: Severe acute pancreatitis; MT: Melatonin treatment.

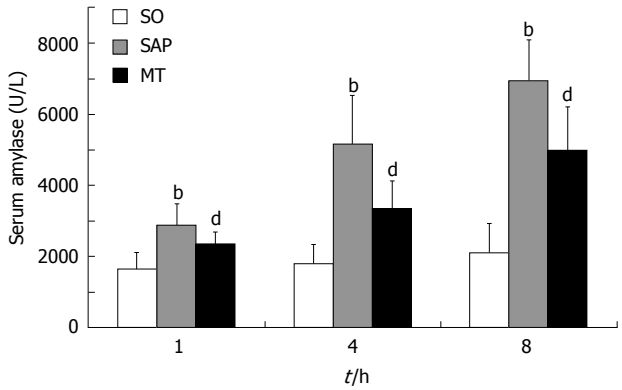


Figure 2 Changes in serum amylase levels from rats in the sham operation, severe acute pancreatitis, and melatonin treatment groups over time. Serum amylase levels differed significantly ($P < 0.01$) between each pair of time points in the SAP group, but not at different levels in the SO group (n = 8 rats per group). $^{b}P < 0.01$ *vs* the SO group; $^{d}P < 0.05$ *vs* the SO and SAP groups. SO: Sham operation; SAP: Severe acute pancreatitis; MT: Melatonin treatment.

the SAP group ($P < 0.01$, Figure 1H).

Serum amylase levels after SAP induction

Levels of serum amylase at each time point were markedly elevated in the SAP group relative to the SO ($P < 0.01$) and MT ($P < 0.05$) groups (Figure 2). In addition, the levels of amylase in the MT group increased compared with those in the SO group. In the SAP group, amylase levels increased over time, indicating the development of SAP. There was no significant difference between the levels of serum amylase at the three time points in the SO group (Figure 2).

Expression of IL-22 and Th22 mRNAs after SAP induction

Levels of the IL-22 and Th22 mRNAs in lung tissues were significantly decreased within 1 h after the induction of SAP ($P < 0.01$; Figure 3A and B), and levels of both mRNAs remained low during the remaining time points. Importantly, this decrease in both mRNAs in the SAP group relative to the SO group was partly rescued in the MT group. Differences between levels in the SAP group and the MT group were significant ($P < 0.01$), and expression differed significantly between the MT group and the SO group ($P < 0.01$, Figure 3A and B).

Levels of IL-22 in lung tissue after SAP induction

The level of IL-22 in lung tissue in the MT group was significantly lower than that in the SO group and significantly higher than that in the SAP group at each time point ($P < 0.01$; Figure 4). The level of IL-22 in the lung tissue samples decreased significantly over time in the SAP group ($P < 0.01$), although IL-22 levels did not differ significantly between each pair of time points in the SO group.

DISCUSSION

Patients with SAP and the associated multiple-organ failure in which it frequently results[20], require admission to a high-dependency or intensive-care unit. Despite recent progress in revealing the mechanisms that underlie SAP, which include inflammation, cell injury, and cell death, the exact pathogenesis of SAP is not fully understood. The uncontrolled generation of reactive oxygen species (ROS) during SAP causes oxidative damage, both in the pancreas and distant organs. The lung is especially vulnerable to damage by ROS because it possesses the largest endothelial surface area of any organ in the body[21].

IL-22 is a member of the IL-10 cytokine family, which additionally comprises IL-10, IL-19, IL-20, IL-24,

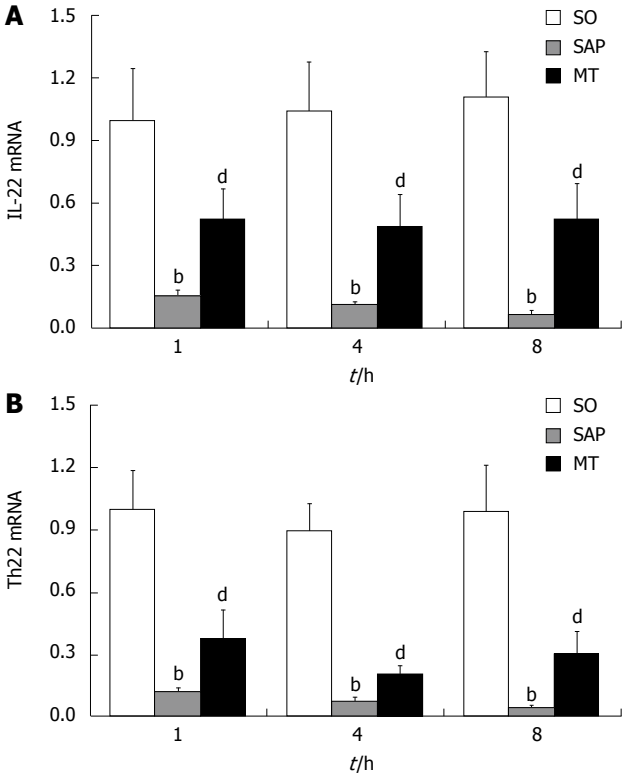


Figure 3 Levels of mRNAs encoding interleukin 22 and T helper cell 22 in lung tissue samples from rats in the sham operation, severe acute pancreatitis, and melatonin treatment groups. A: Expression of IL-22 mRNA 1, 4 and 8 h after induction of SAP; B: Expression of Th22 mRNA 1, 4 and 8 h after induction of SAP. Levels of mRNAs encoding IL-22 and Th22 decreased with continued progression of SAP ($P < 0.01$). $^{b}P < 0.01$ *vs* the SO group; $^{d}P < 0.01$ *vs* the SO and SAP groups ($n = 8$ rats per group). SO: Sham operation; SAP: Severe acute pancreatitis; IL: Interleukin; Th: T helper cell; MT: Melatonin treatment.

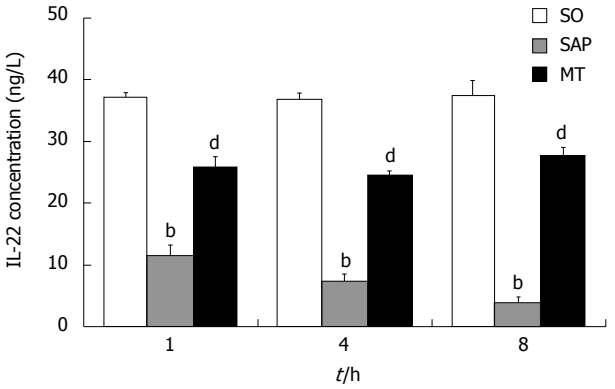


Figure 4 Levels of interleukin 22 in lung tissue samples from rats in the sham operation, severe acute pancreatitis, and melatonin treatment groups. Levels of IL-22 at 1, 4 and 8 h after induction of SAP. Levels of IL-22 decreased with continued progression of SAP ($P < 0.01$). $^{b}P < 0.01$ *vs* the SO group; $^{d}P < 0.01$ *vs* the SO and SAP groups ($n = 8$ rats per group). SO: Sham operation; SAP: Severe acute pancreatitis; IL: Interleukin; Th: T helper cell; MT: Melatonin treatment.

IL-26, IL-28α, IL-28β and IL-29[22-24]. IL-22 is a key player in antimicrobial defenses, tissue regeneration, and protection against damage that may serve either protective or highly pathogenetic roles in chronic inflammatory disorders, depending on the nature of the affected tissue and the local cytokine milieu[5]. IL-22 targets cells from the skin, liver, and kidney and from organs of the respiratory and gastrointestinal systems. The effects of IL-22 in AP are mediated by direct and/or indirect inhibition of the activities and/or production of cycloxygenase-2, prostaglandin E2, IL-1β, and monocyte chemoattractant protein-1, all of which contribute to the progression of SAP[25]. In fact, IL-22 is a potential therapeutic target for the treatment of chronic inflammatory diseases, and treatment with recombinant cytokine or delivery of IL-22 by gene therapy may alleviate tissue destruction during inflammatory responses[26]. Experimental delivery of IL-22 has been efficacious in treating autoimmune disorders such as experimental autoimmune myocarditis in rats[27,28]. Western blotting for IL-22 in bronchoalveolar lavage fluid revealed lower levels of IL-22 in patients with ARDS[29]. This is the first study to suggest the protective actions of IL-22 in a rat model of pancreatitis-associated lung injury.

In this study, we measured the levels of mRNAs encoding IL-22 and Th22 in rat pulmonary tissues to explore the mechanism of acute pancreatitis-associated lung injury. We also examined the effects of SAP, with and without prior treatment with melatonin, on IL-22 protein levels. The reduced levels of IL-22 and Th22 mRNAs that accompanied taurocholate-induction of SAP suggest that downregulation of mRNAs encoding IL-22 and Th22 contribute to the pathogenesis of pancreatitis-associated lung injury. However, future studies are needed to determine more precisely the underlying mechanisms by which downregulation of IL-22 and Th22 contributes to acute pancreatitis-associated lung injury.

Melatonin is a hormone that can cross all body barriers as well as the placenta[30,31]. It is not stored and is rapidly released into the general circulation immediately after its synthesis. Melatonin is useful not only for the acute treatment of ischemic stroke and neurodegenerative disease[32] but also as a prophylactic treatment against stroke[33]. Melatonin is a more highly efficient ROS scavenger than other ROS scavengers and general antioxidants such as glutathione and ascorbic acid, which protect DNA, lipids, and proteins from oxidative damage[34,35]. The previous results have indicated that melatonin prevents or attenuates the severity of experimental SAP[18], but few published studies have examined the relevance between the effect of melatonin and IL-22 or Th22. The main finding of this study was the demonstration that the ameliorative effect of melatonin on pancreatitis-associated lung injury is correlated with an elevation in the levels of IL-22 and Th22, and IL-22 has been demonstrated to protect tissues against caerulein-induced SAP by their anti-inflammatory effects[25].

The levels of mRNAs encoding IL-22 and Th22 were lower in rats with SAP and gradually decreased with the progression of SAP. The levels of IL-22 and Th22 mRNAs in pulmonary tissues from the MT group were, however, partially restored to the levels seen in the SO group, and histopathological examination indicated that the damage to pulmonary tissues from the MT group was reduced relative to that of the SAP group. This sug-

gests that the alleviation of acute pancreatitis-associated lung injury by melatonin is mediated, at least in part, by upregulation of IL-22 and Th22 activities.

In summary, this study indicates, for the first time, that the downregulation of pulmonary IL-22 and Th22 may be associated with the pathogenesis of acute pancreatitis-associated lung injury. Melatonin, functioning as a protectant and antioxidant, may reduce lung injury by increasing IL-22 and Th22 activity.

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COMMENTS

Background

Severe acute pancreatitis (SAP) is a potentially serious disease for which there are limited specific therapies. Pulmonary dysfunction is the most prominent manifestation of extra-abdominal organ dysfunction in pancreatitis. Previous studies assessed the therapeutic efficacy of interleukin 22 (IL-22) and melatonin for the treatment of SAP in a rat model of the disease, although the exact mechanism(s) by which melatonin mediates its effects remains obscure.

Research frontiers

Previous research indicated that therapy involving recombinant IL-22 markedly attenuates the severity of SAP in rats. The study has found that melatonin, which can reduce pancreatic damage in rats with SAP, has a protective effect on the lungs of rats with SAP. This protection is associated with increases in the levels of mRNAs that encode IL-22 and T helper cell 22 (Th22) and increased levels of IL-22 protein. Further research is needed to explore the mechanism by which melatonin upregulates IL-22 and Th22 and how IL-22 and Th22 mediate their protective effects on the lungs of animals with SAP.

Innovations and breakthroughs

The mechanism(s) responsible for the previously proposed anti-inflammatory effects of IL-22 and melatonin during SAP remains to be elucidated. The authors studied the effect of melatonin on levels of mRNAs encoding IL-22 and Th22 and the level of IL-22 in lung tissues from rats with SAP. The results indicated that the downregulation of pulmonary mRNAs encoding IL-22 and Th22 in animals with SAP may be associated with the pathogenesis of acute pancreatitis-associated lung injury, and melatonin is potentially capable of reducing lung injury by increasing IL-22 and Th22 activities. This paper provides a plausible explanation of the mechanism underlying the therapeutic effects of melatonin in the treatment of SAP.

Applications

Given the established therapeutic efficacy of melatonin for treating SAP, elucidation of the underlying mechanism involved is the primary obstacle to translation of this approach to clinical practice. The results suggest that the therapeutic effects of melatonin in reducing acute pancreatitis-associated lung injury can potentially be attributed to increased IL-22 and Th22 activities.

Terminology

SAP is principally caused by autodigestion of the pancreas, and pulmonary dysfunction is the most pertinent manifestation of extra-abdominal organ dysfunction in patients with pancreatitis. IL-22 is a member of the IL10 family of cytokines and represents an important effector molecule of activated IL-22-expressing Th22 cells, Th1, and Th17 cells. Melatonin, which is secreted by the pineal gland, prevents or attenuates the severity of experimental SAP.

Peer review

The authors of this study focus on the value of melatonin in alleviating the severity of acute pancreatitis-associated lung injury and the possible molecular mechanism that underlies this effect. The results suggest that the down-regulation of pulmonary IL-22 and Th22 may be associated with the pathogenesis of acute pancreatitis-associated lung injury and that melatonin is potentially capable of reducing lung injury by increasing IL-22 and Th22 activity.

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BRIEF ARTICLE

CpG island methylator phenotype and *Helicobacter pylori* infection associated with gastric cancer

Ji-Bin Liu, Xu-Ming Wu, Jin Cai, Jin-Ye Zhang, Jin-Lin Zhang, Shu-Hui Zhou, Min-Xin Shi, Fu-Lin Qiang

Ji-Bin Liu, Xu-Ming Wu, Jin Cai, Jin-Ye Zhang, Jin-Lin Zhang, Min-Xin Shi, Fu-Lin Qiang, Tumor Institute, Nantong Tumor Hospital, Nantong 226361, Jiangsu Province, China
Shu-Hui Zhou, School of Radiology and Public Health, Soochow University, Suzhou 215123, Jiangsu Province, China
Author contributions: Liu JB and Wu XM contributed equally to this work; Liu JB and Qiang FL designed the study, performed data analysis and interpretation; Cai J, Zhang JY and Zhang JL critically revised the manuscript for intellectual content; Wu XM and Zhou SH performed the statistical analysis; Shi MX was responsible for data acquisition.
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Correspondence to: Dr. Fu-Lin Qiang, Tumor Institute, Nantong Tumor Hospital, 30 Tongyang Beilu, Pingchao Town, Nantong 226361, Jiangsu Province, China. doctorjibinliu@163.com
Telephone: +86-513-86729067 Fax: +86-513-86571201
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Abstract

AIM: To investigate the association between the CpG island methylator phenotype (CIMP) and serum *Helicobacter pylori* (*H. pylori*) levels for clinical prediction of gastric cancer (GC) progression.

METHODS: We analyzed the serum CIMP status of 75 patients with GC using a methylation marker panel and a methylation-specific polymerase chain reaction. Serum samples from 40 healthy persons were examined at the same time. The genes examined were *APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK* and *E-cad*. *H. pylori* infection in serum was assayed with an anti-*H. pylori* immunoglobulin G antibody test and a rapid urease test.

RESULTS: The frequencies of high-level methylation in GC tissues for the seven genes were: 48% for *APC*, 57.33% for *WIF-1*, 56% for *RUNX-3*, 50.67% for *DLC-1*, 52% for *SFRP-1*, 54.67% for *DKK*, and 48% for *E-cad*. The frequencies in GC serum were 30.67% for *APC*, 34.67% for *WIF-1*, 37.33% for *RUNX-3*, 29.33% for *DLC-1*, 33.33% for *SFRP-1*, 32% for *DKK*, and 26.67% for *E-cad*. CIMP+ (defined as $\geqslant$ 3 methylated genes) was associated with 47 (62.67%) GC tissue samples and 44 (58.67%) GC serum samples. CIMP+ was not associated with non-neoplastic mucosal tissues or the serum of healthy persons. Of the 75 GC cases, 51 (68%) were *H. pylori*+, and 24 (32%) were *H. pylori*-. Of the 51 *H. pylori*+ cases, 36 were CIMP+ and 15 were CIMP-. In contrast, for the 24 *H. pylori*- cases, 11 were CIMP+, and 13 were CIMP-. The difference was significant between the *H. pylori*+ and *H. pylori*- groups (χ^2 = 4.27, P < 0.05). Of the 51 *H. pylori*+ GC patients, 34 were CIMP+ and 17 were CIMP-, while among the 24 *H. pylori*- GC cases, 10 were CIMP+ and 14 were CIMP-. The difference was significant between the *H. pylori*+ and *H. pylori*- groups (χ^2 = 4.21, P < 0.05). A 2-year follow-up showed significant difference in the rates of metastasis and recurrence between *H. pylori*+/CIMP+ cases and the *H. pylori*+/CIMP- cases or CIMP- cases associated with *H. pylori* assayed in serum (P < 0.05). However, there were no significant differences in survival rates between the two groups.

CONCLUSION: *H. pylori*+/CIMP+ cases are associated with higher rates of metastasis and recurrence than *H. pylori*+/CIMP- cases. Serum may be useful for examining CIMP status.

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Key words: CpG island methylator phenotype; *Helicobacter pylori*; Serum; Prognosis; Gastric cancer

Peer reviewer: Ki-Baik Hahm, MD, PhD, Professor, Gachon Graduate School of Medicine, Department of Gastroenterology, Lee Gil Ya Cancer and Diabetes Institute, Lab of Translational Medicine, 7-45 Songdo-dong, Yeonsu-gu, Incheon 406-840, South Korea

Liu JB, Wu XM, Cai J, Zhang JY, Zhang JL, Zhou SH, Shi

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INTRODUCTION

Gastric cancer (GC) is one of the most common human tumors and is the second-leading cause of cancer-related deaths worldwide[1]. GC remains a major clinical challenge because it has a poor prognosis and limited treatment options due to its relative resistance to radiotherapy and chemotherapy[2].

DNA methylation is an enzyme-mediated chemical modification that occurs in cytosine-guanine dinucleotide-rich areas (CpG islands) in gene promoter regions. Aberrant promoter hypermethylation leads to loss of gene function in tumors[3]. In several types of cancers, including GC, global hypomethylation and promoter hypermethylation in specific genes are associated with genomic instability and inactivation of tumor-suppressor genes[4]. The methylation pattern(s) of multiple genes can provide useful information regarding global epigenetic alterations[5]. The hypermethylated subtype associated with tumors, denoted that the CpG island methylator phenotype (CIMP) for which multiple genes are concurrently methylated, is a novel marker of tumor progression[6-8].

Helicobacter pylori (*H. pylori*) infection of the stomach is associated with an increased risk for gastric carcinoma[9]. Several studies have demonstrated that *H. pylori* infection is associated with gene promoter hypermethylation and gene-type-specific methylation profiles involved in the multistep process of carcinogenesis[10,11]. *H. pylori* infection may induce methylation of the Trefoil factor family 2 and E-cadherin promoters in GC[12]. Certain studies have shown that methylation levels in GC patients are one to two orders of magnitude greater in *H. pylori*+ individuals than in *H. pylori*- individuals[13]. In infected mucosae, aberrant methylation and subsequent silencing of tumor suppressor genes, including *p16*, *E-cadherin*, and *hMLH1*, are strongly correlated with subsequent cancer risk[14].

In the present study, we focused on the relationship between *H. pylori* infection and CIMP status in GC risk. We did not specifically study the relationship between CIMP and *H. pylori* infection or the clinical value of evaluating CIMP associated with *H. pylori* infection.

We analyzed the relationship between serum CIMP of 75 GC patients and *H. pylori* infection in GC. The examined genes were *APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK*, and *E-cad*, which were selected because they had been found to be frequently methylated in GC and other malignancies The aim of this study was to evaluate the clinical significance of serum CIMP and *H. pylori*-infection levels for the prediction of GC progression.

MATERIALS AND METHODS

Patients and specimens

Between 2008 and 2009, tumor samples, adjacent non-neoplastic tissues, and sera from 75 Chinese GC patients were collected during surgical resection at the Nantong Tumor Hospital. Samples from 40 healthy people were used as matched controls. The controls had no self-reported history of cancer and could frequently be matched to the cases in age (± 5 years), gender, and residential area. After resection, tissue specimens were immediately frozen in liquid nitrogen and stored at -70 ℃. Patients consisted of 53 men and 22 women, ranging in age from 31 to 76 (52 ± 7) years. Questionnaire data and blood samples were also collected from all patients and controls. After centrifugation, isolated serum samples were stored at -70 ℃. The diagnosis of GC was confirmed by pathological examination and magnetic resonance imaging and/or computerized tomography. Tumor-node-metastasis (TNM) stage was classified according to the 6th edition TNM classification of the American Joint Committee on Cancer. Written informed consent was obtained from each patient and healthy control, and the local ethics committee approved the study protocol.

DNA extraction and sodium bisulfite

DNA from serum (200 μL per sample) and tissue samples was treated with proteinase K and then extracted with phenol-chloroform according to the manufacturer's instructions (Shanghai ShineGene Molecular Biotech, Inc., Shanghai, China) and stored at -20 ℃. A final elution volume of 50 μL was collected. The extracted DNA was treated with sodium bisulfite using EZ DNA Methylation kit reagents (Zymo Research, Orange, CA, United States) to convert all unmethylated cytosines to uracils. Bisulfite-modified DNA was suspended in 10 μL of elution buffer and stored at -20 ℃ until methylation-specific polymerase chain reaction (PCR) (MSP) was performed.

MSP

MSP was used to determine the methylation status of CpG islands in genes after bisulfate treatment[15,16]. The methylation status of the promoters of *APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK*, and *E-cad* was determined with a two-step amplification/detection MSP protocol. PCR products were electrophoresed through 2% agarose gels, stained with ethidium bromide, and visualized with ultraviolet illumination. All experiments were performed in duplicate. Table 1 lists the sequences of the PCR primers used.

H. pylori assay

H. pylori infection was assayed with the reagents of a serum anti-*H. pylori* immunoglobulin G (IgG) antibody test (PBM, PRINCETON, United States) and a rapid urease test (Fujian Sanqiang Biological and Chemical Co., Ltd,

Table 1 Primer sets for nested methylation-specific polymerase chain reaction

Gene			Primer sequence (5'→3')
APC	U	F	GTGTTTTATTGTGGAGTGTGGGTT
		R	CCAATCAACAAACTCCCAACAA
	M	F	TATTGCGGAGTGCGGGTC
		R	TCGACGAACTCCCGACGA
WIF-1	U	F	GGGTGTTTTATTGGGTGTATTGT
		R	AAAAAAACTAACACAAACAAAATACAAAC
	M	F	CGTTTTATTGGGCGTATCGT
		R	ACTAACGCGAACGAAATACGA
RUNX-3	U	F	TTATGAGGGGTGGTTGTATGTGGG
		R	AAAACAACCAACACAAACACCTCC
	M	F	TTACGAGGGGCGGTCGTACGCGGG
		R	AAAACGACCGACGCGAACGCCTCC
DLC-1	U	F	AAACCCAACAAAAAAACCCAACTAACA
		R	TTTTTTAAAGATTGAAATGAGGGAGTG
	M	F	CCCAACGAAAAAACCCGACTAACG
		R	TTTAAAGATCGAAACGAGCGAGCG
SFRP-1	U	F	GAGTTAGTGTTGTGTGTTTGTTGTTTTGT
		R	CCCAACATTACCCAACTCCACAACCA
	M	F	GTGTCGCGCGTTCGTCGTTTCGC
		R	AACGTTACCCGACTCCGCGACCG
DKK	U	F	TTAGGGGTGGGTGGTGGGGT
		R	CTACATCTCCACTCTACACCCA
	M	F	GGGGCGGGCGGCGGGGC
		R	ACATCTCCGCTCTACGCCCG
E-cad	U	F	TAATTTTAGGTTAGAGGGTTATTGT
		R	CCACCCCAATACTAAATCACAACA
	M	F	TTAGGTTAGAGGGTTATCGCGT
		R	TAACTAAAAATTCACCTACCGAC

M: Methylated sequence; U: Unmethylated sequence; F: Forward sequence; R: Reverse sequence.

Sanming, China). The sensitivities of the serum anti-*H. pylori* IgG antibody test and rapid urease test have been reported to be ≥ 90% of the culture test[13].

Follow-up examination

Patients were followed up to 2 years after surgery. The last follow-up was on September 12, 2010. Patients had not received chemotherapy prior to surgery. The median follow-up period was 17.5 mo (range, 8-28 mo). Patients were given a physical examination, abdominal ultrasonography, and chest X-ray, and serum was collected and tested for associated tumor markers. During the first year, local recurrence and the development of distant metastasis were monitored every 3 mo with computerized tomography and/or magnetic resonance imaging. Of the 75 patients, 13 (18.67%) died from cancer-related causes. We tracked all but three of the remaining patients during the entire study period. Seventeen patients (22.67%) had GC recurrence found on computerized tomography Fourteen patients (18.67%) had GC metastases. Sixty-two patients were still alive at the time of the last follow-up report.

Statistical analysis

The SPSS 13.0 software package (SPSS, Inc., Chicago, IL, United States) was used for statistical analysis. Values for the clinical and biological characteristics of the patients are expressed as mean ± SD. Comparison was done with the Student's *t* test. The χ^2 test was used to compare the incidence of methylation. All *P* values are two-sided, and a *P* value < 0.05 was considered statistically significant.

Table 2 Number of samples with methylated genes for gastric cancer, adjacent non-neoplastic tissue, and serum from gastric cancer patients and healthy controls

Gene	Tumor tissue (*n* = 75)	Serum from GC patients (*n* = 75)	Non-neoplastic tissues (*n* = 72)	Serum from healthy controls (*n* = 40)	χ^2
APC	36[a]	23	3	0	36.21
WIF-1	43[a]	26	4	0	45.28
DLC-1	38[a]	22	3	0	39.49
DKK	41[a]	24	2	0	47.79
SFRP-1	39[a]	25	4	0	38.29
E-cad-1	36[a]	20	3	0	36.21
RUNX-3	42[a]	28	5	0	40.64

[a]*P* < 0.05 *vs* the number of methylated gene promoters for tumor and non-neoplastic tissue samples with the χ^2 test. GC: Gastric cancer.

RESULTS

Clinical data

The median tumor size was 3.8 cm (range 2.5-9.6 cm). According to the Edmondson-Steiner classification system, six cases were classified as grade Ⅰ, 22 as grade Ⅱ, 40 as grade Ⅲ, and seven as grade Ⅳ. According to the 6th edition TNM classification of the American Joint Committee on Cancer, 24 cases were classified as grade Ⅰ, 31 as grade Ⅱ, and 20 as grade Ⅲ.

Methylation of tumor-associated genes in GC

We examined the methylation status of seven tumor-associated genes (*APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK* and *E-cad*) from tissue and serum samples of GC patients and from serum samples of controls. The overall results are summarized in Table 2. No methylated gene promoters from control serum were detected. Methylation occurred more frequently in the GC DNA than in adjacent non-neoplastic mucosal tissues, and the same relationship was observed for serum samples and non-neoplastic tissues. Methylation was detected in one or more of the genes in 69 (92%) of the 75 cases. The frequencies of high-level methylation in GC tissue and serum were ≥ 15% for the seven genes: 48% for *APC*, 57.33% for *WIF-1*, 56% for *RUNX-3*, 50.67% for *DLC-1*, 52% for *SFRP-1*, 54.67% for *DKK*, and 48% for *E-cad* in tissue. In serum, the frequencies were 30.67% for *APC*, 34.67% for *WIF-1*, 37.33% for *RUNX-3*, 29.33% for *DLC-1*, 33.33% for *SFRP-1*, 32% for *DKK*, and 26.67% for *E-cad*.

According to the criteria used in a related study[17], the CIMP status of our 75 GC samples was classified as CIMP+ (≥ 3 methylated genes) or CIMP- (two or fewer methylated genes). In this study, because the cutoff value was 3, the average number of methylated genes found

Table 3 CpG island methylator phenotype associated with *Helicobacter pylori* infection in 75 gastric cancer tissue and serum samples

CIMP	*H. pylori* infection in tissue samples		*H. pylori* infection in serum samples	
	Negative (-)	Positive (+)	Negative (-)	Positive (+)
Negative (-)	13	15	14	17
Positive (+)	11	36[a]	10	34[a]

[a]$P < 0.05$ *vs* the frequencies of CIMP between *H. pylori*+ and *H. pylori*- samples in serum with the χ^2 test. *H. pylori*: *Helicobacter pylori*; CIMP: CpG island methylator phenotype.

was 3.6 in tumor tissue and 2.0 in serum. For tumor tissue samples, 47 (62.67%) were classified as CIMP+, and 28 (37.33%) were classified as CIMP-. For serum samples, 44 (58.67%) were classified as CIMP+, and 31 (41.33%) were classified as CIMP-. No non-neoplastic tissues or serum from healthy controls were classified as CIMP+.

H. pylori in GC

We determined how many of the 75 GC patients were infected with *H. pylori* and found 51 (68%) to be *H. pylori*+ and 24 (32%) to *H. pylori*-.

Of the 51 *H. pylori*+ GC cases examined in tissues, 36 were CIMP+ and 15 were CIMP-. In contrast, of the 24 *H. pylori*- GC cases, 11 cases were CIMP+ and 13 were CIMP-. There was a significant difference between the *H. pylori*+ and *H. pylori*- groups ($\chi^2 = 4.27$, $P < 0.05$). When the sera from the 51 *H. pylori*+ GC cases were examined, 34 were CIMP+ and 17 were CIMP-. In contrast, of the 24 *H. pylori*- GC cases, 10 were CIMP+ and 14 were CIMP-. There was a significant difference between the two groups ($\chi^2 = 4.21$, $P < 0.05$). The overall results are summarized in Table 3.

Prognostic significance of CIMP associated with H. pylori infection in GC

After the two-year follow-up, the metastasis rates were found significantly different between the *H. pylori*+/CIMP+ and the *H. pylori*+/CIMP- cases when analyzed in serum ($P < 0.05$). The tumors of *H. pylori*+/CIMP+ patients frequently metastasized. The recurrence rates were significantly higher in the *H. pylori*+/CIMP+ group than in the *H. pylori*+/CIMP- group when serum was examined ($P < 0.05$). There were no differences in survival rates between *H. pylori*+/CIMP+ cases and *H. pylori*+/CIMP- cases examined in serum ($P > 0.05$). The overall results are summarized in Table 4.

Table 4 Prognosis of CpG island methylator phenotype cases associated with *Helicobacter pylori* infection in gastric cancer

Prognosis	*H. pylori*+		*H. pylori*-		χ^2
	CIMP+	CIMP-	CIMP+	CIMP-	
Metastasis					
Yes	9	2	2	1	3.593[a]
No	22	15	8	13	
Recurrence					
Yes	10	3	2	2	2.369[a]
No	21	14	8	12	
Survival					
Yes	24	15	8	12	1.043
No	7	2	2	2	

[a]$P < 0.05$ *vs* the prognosis between *H. pylori*+/CIMP+ and *H. pylori*+/CIMP- in serum with the χ^2 test. *H. pylori*: *Helicobacter pylori*; CIMP: CpG island methylator phenotype.

DISCUSSION

Epigenetic alterations have been suggested to be significant initiating events in tumorigenesis[18]. Aberrant methylation of promoter DNA regions rich in CpG islands is the key step in epigenetic gene silencing. Abnormal gene expression may be an early event in tumorigenesis and a potential biomarker for early detection[19]. In several studies, aberrant DNA methylation in gastric biopsies from *H. pylori*+ patients was found to be correlated with a greater gastric cancer risk[13,20]. Methylation level of promoters in several genes in the gastric mucosa is 5-300 times higher in infected than in uninfected individuals. In infected mucosae, the aberrant methylation and subsequent silencing of tumor suppressor genes has been strongly correlated with subsequent cancer risk[14].

Notably, the term "CIMP" has been used in a variety of ways in the context of gastric carcinoma[21]. The hypermethylator phenotype may be related to patient-specific factors, such as exposure to carcinogens or genetic predisposition[22]. The relationship between *H. pylori* and methylation of multiple genes has been demonstrated, but little attention has been paid to the association of CIMP and *H. pylori* with respect to gastric cancer or the relationship between CIMP in serum and the presence of *H. pylori* in gastric cancer.

We collected tissue and serum samples from GC patients and healthy controls to examine the methylation status of seven tumor-associated genes (*APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK*, and *E-cad*). We found that the promoters of these genes from healthy control serum were not methylated. The frequencies of high-level methylation in GC tissue and serum were at least 15% for these seven genes. Thus, examining the methylation status of multiple genes may help diagnose early GC. We also found that promoter methylation in serum DNA was highly specific for the cancer-related genes and that the results in serum samples were similar to tissue samples. We also determined the CIMP status of 75 GC samples and found a good concordance between serum and tumor CIMP status.

Of the 75 GC cases, 51 (68%) were *H. pylori*+ and 24 (32%) were *H. pylori*-. Thus, *H. pylori* infection was substantial in GC, suggesting that eradicating *H. pylori* infection may be essential to prevent or treat GC.

The prognostic roles of CIMP status have been evaluated in several cancer types. In esophageal adenocarcinoma, CIMP is associated with a poor prognosis[16]. In GC, concordant methylation of multiple genes/loci (CIMP-H)

is associated with a better survival, but is not an independent predictor of prognosis in resected GC[2].

H. pylori infection is also a factor in the prognosis of GC. We found that CIMP was associated with *H. pylori* infection. At the end of the 2-year follow-up, we found that the rates of metastasis differed significantly between *H. pylori*+/CIMP+ and *H. pylori*+/CIMP- cases when examining the serum ($P < 0.05$). The tumors of *H. pylori*+/CIMP+ cases frequently metastasized. The recurrence rates were also significantly higher in the *H. pylori*+/CIMP+ cases than in the *H. pylori*+/CIMP- cases when serum was examined ($P < 0.05$). There were no significant differences in survival rates between *H. pylori*+/CIMP+ and *H. pylori*+/CIMP- cases examined in serum ($P > 0.05$). Therefore, aberrant DNA hypermethylation may be frequently associated with chronic inflammation, but not with *H. pylori* infection. Modulation levels of pleiotropic regulators suggested that DNA methylation plays an important role in host response to *H. pylori* infection. *H. pylori* promotes genetic instability via decreasing expression of DNA repairing genes[22,23]. We observed that CIMP+ in conjunction with *H. pylori* infection was associated with metastasis and recurrence of GC, but not the survival rate after the 2-year follow-up period.

In summary, our study shows that promoter methylation in serum DNA was highly specific and that serum and tissue samples yielded similar results. Therefore, CIMP detection in serum, rather than in tumor tissues, may be used as a reliable index for predicting the clinical course of patients with GC.

This study is limited by a relatively small number of patients, cancer-related genes, as well as follow-up period of less than 2 years. Thus, larger-scale multi-gene studies with an extended follow-up period are needed to validate our results.

COMMENTS

Background

Gastric cancer (GC) is one of the most common human tumors and is the second-leading cause of cancer-related deaths worldwide. GC remains a major clinical challenge because it has a poor prognosis and limited treatment options due to its relative resistance to radiotherapy and chemotherapy. Aberrant DNA methylation in gastric biopsies from *Helicobacter pylori* (*H. pylori*)+ patients has been shown to be correlated with a greater GC risk. The relationship between *H. pylori* and methylation of multiple genes has been demonstrated, but little attention has been paid to the association of the CpG island methylator phenotype (CIMP) and *H. pylori* with respect to GC or the relationship between CIMP in serum and the presence of *H. pylori* in GC.

Research frontiers

DNA methylation is an enzyme-induced chemical modification that occurs in cytosine-guanine dinucleotide-rich areas (CpG islands) in the gene promoter regions. Aberrant promoter hypermethylation is an important mechanism for loss of gene function in tumors including GC. The methylation pattern of multiple genes can provide useful information and an overall picture of epigenetic alterations. The hypermethylated subtype in tumors is termed as the CIMP, where multiple genes are concurrently methylated. *H. pylori* infection is found associated with gene promoter hypermethylation and gene-type-specific methylation profiles that are involved in the multistep process of carcinogenesis. Methylation levels in patients with GC are one to two orders of magnitude greater in *H. pylori*+ individuals than in *H. pylori*- individuals.

Innovations and breakthroughs

In the present study, the authors focused on the relationship between *H. pylori* infection and CIMP status in GC risk. The authors analyzed the relationship between serum CIMP of 75 patients with GC and *H. pylori* infection in GC. The examined genes were *APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK*, and *E-cad*, which were selected because they had been found to be frequently methylated in GC and other malignancies. This study evaluated the clinical significance of serum CIMP and *H. pylori*-infection levels for the prediction of GC progression.

Applications

It was found in this study that *H. pylori*+/CIMP+ cases were associated with higher metastasis and recurrence rates than *H. pylori*+/CIMP- cases. CIMP detection in serum, rather than in tumor tissues, may be used as a reliable index for predicting the clinical course of patients with GC.

Terminology

Epigenetic changes: Heritable changes in gene structure without changing the gene sequence; CpG islands: CpG rich areas located in the promoter regions of many genes; CpG island methylation: The addition of a methyl group to a cytosine residue that lies next to guanine within CpG dinucleotides; CIMP: The hypermethylated subtype in tumors, where multiple genes are concurrently methylated.

Peer review

This manuscript was well designed and executed, and data were summarized well.

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CASE REPORT

Use of anti tumor necrosis factor-alpha monoclonal antibody for ulcerative jejunoileitis

Gulseren Seven, Adel Assaad, Thomas Biehl, Richard A Kozarek

Gulseren Seven, Richard A Kozarek, Digestive Disease Institute, Virginia Mason Medical Center, Seattle, WA 98101, United States
Adel Assaad, Department of Pathology, Virginia Mason Medical Center, Seattle, WA 98101, United States
Thomas Biehl, Department of General, Thoracic and Vascular Surgery, Virginia Mason Medical Center, Seattle, WA 98101, United States
Author contributions: Seven G and Kozarek RA designed and performed research; Seven G and Kozarek RA wrote and edited the paper; Assaad A and Biehl T contributed acquisition and interpretation of data and writing.
Correspondence to: Richard A Kozarek, MD, Digestive Disease Institute, Virginia Mason Medical Center, 1100 9th Ave., PO Box 900, Mail Stop C3-GAS, Seattle, WA 98101, United States. gasrak@vmmc.org
Telephone: +1-206-2232310 Fax: +1-206-3410061
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Abstract

Ulcerative jejunoileitis is an uncommon clinical syndrome consisting of abdominal pain, weight loss associated with diarrhea, and multiple inflammatory ulcerations and strictures of the small bowel. Ulcerative jejunoileitis can complicate established celiac disease or develop in patients *de novo*. Increased levels of tumor necrosis factor-alpha (TNF-α) in the small intestine of patients with untreated celiac disease are associated with a role in the immune pathogenesis of this disorder. No specific therapy has been shown to change the course of ulcerative jejunoileitis. We report a case of severe ulcerative jejunoileitis previously unresponsive to traditional therapies, including high dose corticosteroids and cyclosporine. The patient had a dramatic resolution of symptoms and a complete normalization of endoscopic findings after anti-TNF-α monoclonal antibody, infliximab (Remicade®).

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Key words: Ulcerative jejunoileitis; Biologic therapy; Tumor necrosis factor-alpha; Infliximab

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INTRODUCTION

Ulcerative jejunoileitis, also termed chronic nongranulomatous enteritis or idiopathic chronic ulcerative jejunoileitis, is a clinical syndrome consisting of abdominal pain, diarrhea, and multiple inflammatory ulcerations of the small bowel, especially the jejunum[1]. Ulcerative jejunoileitis can complicate established celiac disease or occur in patients *de novo*[1,2]. It is uncommon and largely a diagnosis of exclusion.

CASE REPORT

A 62-year-old man presented in May 2009 with a 20-year history of intermittent abdominal pain, diarrhea, weight loss, and gastrointestinal bleeding. In 2008, he was admitted at another institution with abdominal pain, and found to have a perforated jejunum with an interloop abscess requiring small bowel resection. There was no evidence of granulomas or histologic Crohn's disease in the resected specimen, and no history of diverticulum at the site of perforation. He was hospitalized several times with pain, bleeding, and intussusception in the postoperative period. He had been started on 25 mg prednisone per day preoperatively because of possible Crohn's disease and continued on this medication.

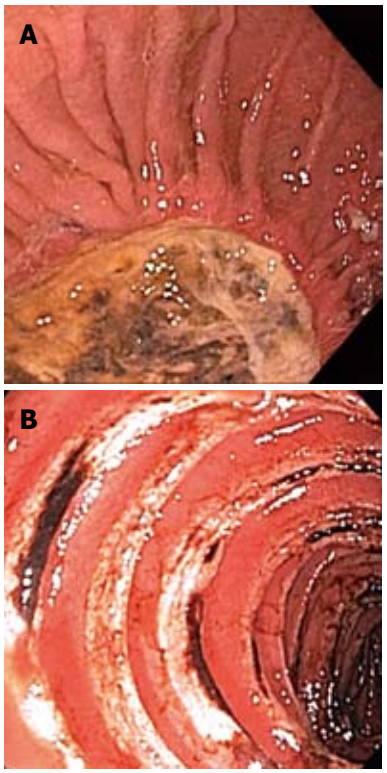


Figure 1 Enteroscopic appearance of the lesions. A: A 4-cm chronic cavity consistent with chronic perforation at the jejunal anastomosis; B: More distal jejunal ulcerations.

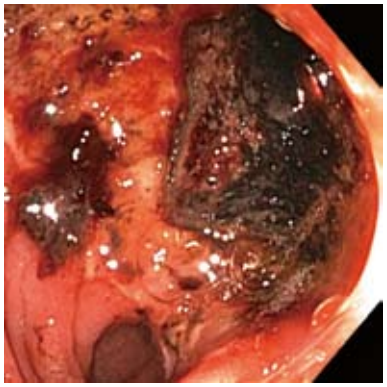


Figure 2 Endoscopy showing bleeding from an anastomotic ulcer.

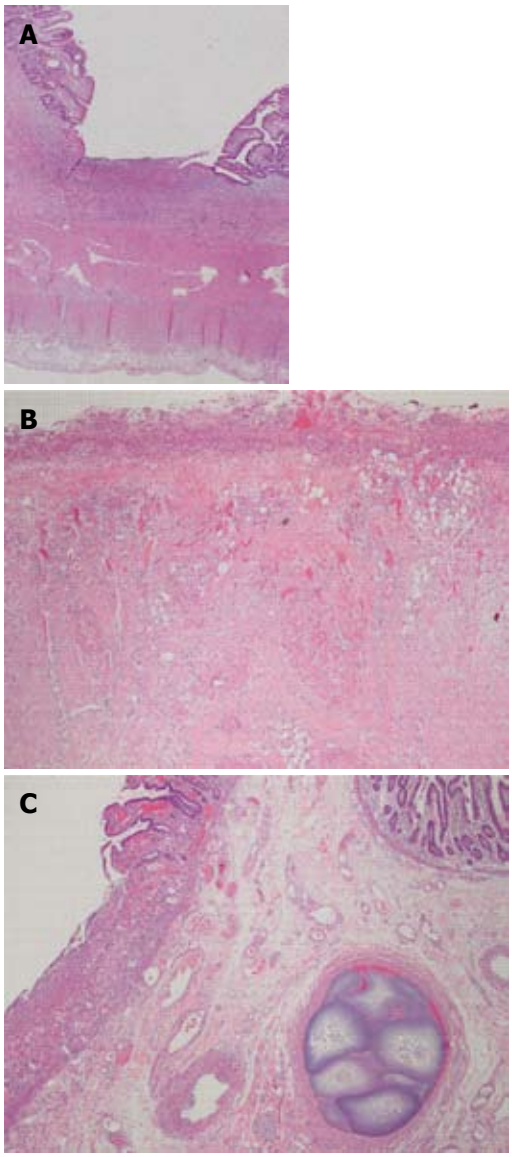


Figure 3 Surgically resected jejunum. A: Full thickness small bowel section showing ulcerated small intestinal mucosa; B: Ulcer bed showing acute and chronic inflammation, granulation tissue and overlying fibrinopurulent exudate, no granulomas are seen; C: Intravascular embolization material in submucosal blood vessel.

Physical examination revealed a chronically ill, tremulous man. His prednisone was increased to 40 mg/d, but the patient continued to have abdominal pain and bloody bowel movements. Enteroscopy showed circumferential ulceration and a stricture of the distal esophagus with a clot, severe duodenal ulceration, a stricture and a 4-cm ulcer cavity at the jejunal anastomosis as well as more distal ulceration (Figure 1A and B). Computerized tomography (CT) of the abdomen demonstrated a long segment of inflamed jejunum and mesenteric adenopathy. Serum gastrin level was normal. Despite parenteral nutrition, 60 mg/d of prednisone and 8 mg/kg per day of oral cyclosporine, the patient continued to have refractory diarrhea and bleeding. Repeat endoscopies demonstrated deep anastomotic and multiple distal ulcerations.

Because of relapsing bleeding and steroid-dependence, infliximab was initiated at 5 mg/kg dose at 0, 2 and 6 wk, followed by every eight-week intervals in conjuction with cyclosporine and prednisone. One month after initial infusion, he was admitted with rebleeding from the anastomotic ulcer (Figure 2). Angiography was negative for active bleeding. An exploratory laparotomy was performed with distal duodenal and proximal jejunal resection and revealed a chronic 5 cm jejunal ulcer at the jejunojejunostomy site associated with microperforation and an inflammatory mass with matted lymph nodes. Full thickness biopsies of the jejunum demonstrated multiple ulcerations but no features of Crohn's, celiac disease, ischemia, lymphoma, vasculitis, neoplasm, or infectious etiology (Figure 3A-C). After the surgery, prednisone was

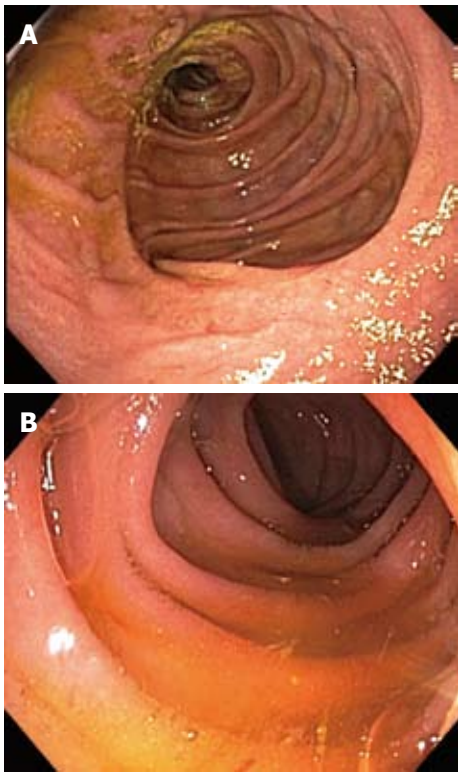


Figure 4 Complete improvement on endoscopic findings after infliximab therapy. A: Jejunojejunostomy; B: Distal jejunum.

tapered and discontinued 6 mo postoperatively.

To date (30 mo), the patient is stable on every eight week infliximab infusion. His PPI has been discontinued, and there is no evidence of ulceration, enteritis, or lymphoma on follow-up enteroscopy (Figure 4A and B), colonoscopy, or abdominal CT.

DISCUSSION

Although pathophysiology of ulcerative jejunoileitis is poorly understood, it may be related to mucosal infiltration by activated T-cells. A molecular analysis of mucosal T-cell populations has previously shown that monoclonal T-cells exist in ulcers and normal mucosa, suggesting a fundamental role of T-cells in ulcer pathogenesis[3]. Another study demonstrated that the cytotoxic capability of T-cells is responsible for the development of the ulcers with preference for mucosa-associated lymphoid tissue localization[4]. Tumor necrosis factor alpha (TNF-α) plays a pivotal role in Crohn's disease[5]. Increased levels of interleukin-6 and TNF-α in the lamina propria and epithelium of patients with untreated celiac disease also support their role in the development of the mucosal damage observed in celiac disease[6]. Infliximab therapy results in a reduction in the activated T lymphocytes that accumulate within the lamina propria in Crohn's disease, and neutralization of TNF is effective in induction and maintenance of remission in Crohn's disease[7]. Case reports using infliximab in patients with refractory celiac disease have shown improvement in symptoms, and duodenal histology[8,9]. One additional case of ulcerative jejunoileitis complicating refractory celiac disease has been published, demonstrating improvement in fecal output and small bowel histology[10]. However, a complete recovery was not achieved and parenteral nutritional support continued. Seven months later, the patient developed a stenosis of the distal ileum and surgical resection demonstrated enteropathy-associated T-cell lymphoma.

In contrast, we have demonstrated that infliximab plays a key role in the management of ulcerative jejunoileitis and should be considered a treatment option for patients with this disorder.

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CASE REPORT

Scrub typhus hepatitis confirmed by immunohistochemical staining

Jong-Hoon Chung, Sung-Chul Lim, Na-Ra Yun, Sung-Heui Shin, Choon-Mee Kim, Dong-Min Kim

Jong-Hoon Chung, Na-Ra Yun, Dong-Min Kim, Department of Internal Medicine, Chosun University College of Medicine, Gwangju 501-717, South Korea
Sung-Chul Lim, Sung-Heui Shin, Choon-Mee Kim, Dong-Min Kim, Research Center for Resistant Cells, Chosun University, Gwangju 501-717, South Korea
Sung-Chul Lim, Department of Pathology, Chosun University College of Medicine, Gwangju 501-717, South Korea
Sung-Heui Shin, Department of Microbiology, Chosun University College of Medicine, Gwangju 501-717, South Korea
Author contributions: Chung JH and Lim SC contributed equally to this work; Chung JH, Lim SC and Kim DM conceived, designed and wrote the paper; Yun NR, Shin SH and Kim CM contributed reagents/materials/analytic tools.
Supported by The Korea Science and Engineering Foundation Grant Funded by the Korean Government through the Research Center for Resistant Cells, No. R13-2003-009
Correspondence to: Dong-Min Kim, Professor, Department of Internal Medicine, Chosun University College of Medicine, 588 Seosuk-dong, Dong-gu, Gwangju 501-717, South Korea. drongkim@chosun.ac.kr
Telephone: +82-62-2203108 Fax: +82-62-2349653
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Abstract

Scrub typhus is an acute febrile disease caused by *Orientia tsutsugamushi* (*O. tsutsugamushi*). We report herein the case of a woman who presented with fever and elevated serum levels of liver enzymes and who was definitively diagnosed with scrub typhus by histopathological examination of liver biopsy specimens, serological tests and nested polymerase chain reaction. Immunohistochemical staining using a monoclonal anti-*O. tsutsugamushi* antibody showed focally scattered positive immunoreactions in the cytoplasm of some hepatocytes. This case suggests that scrub typhus hepatitis causes mild focal inflammation due to direct liver damage without causing piecemeal necrosis or interface hepatitis. Thus, scrub typhus hepatitis differs from acute viral hepatitis secondary to liver damage due to host immune responses, which causes severe lobular disarray with diffuse hepatocytic degeneration, necrosis and apoptosis as well as findings indicative of hepatic cholestasis, such as hepatic bile plugs or brown pigmentation of hepatocytes.

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Key words: Scrub typhus; Immunohistochemistry; *Orientia tsutsugamushi*

Peer reviewer: Jian-Zhong Zhang, Professor, Department of Pathology and Laboratory Medicine, Beijing 306 Hospital, 9 North Anxiang Road, PO Box 9720, Beijing 100101, China

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INTRODUCTION

Scrub typhus is an acute febrile disease caused by *Orientia tsutsugamushi* (*O. tsutsugamushi*) that occurs when *O. tsutsugamushi* is transmitted to humans through the bites of chiggers (*Leptotrombidium*)[1]. Previous reports from Taiwan have revealed that 97% of hospitalized scrub typhus patients have high serum levels of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase as well as subsequent abnormal liver function[2,3]. However, only a few studies have evaluated the histopathological features of patients with scrub typhus hepatitis[4]. We report herein the case of a woman who presented with fever and elevated serum levels of liver enzymes and who was definitively diagnosed with scrub typhus by a histopathological examination of liver biop-

sy specimens, serological tests and a nested polymerase chain reaction (PCR).

CASE REPORT

A 63-year-old woman presented at our clinic with a 5-d history of fever. Five days previously, the patient had visited a primary care clinic due to fever, chills, headache, myalgia, anorexia and diarrhea. She was transferred to our clinic because of her unusually high serum levels of liver enzymes and low platelet count. She was a housewife by occupation and had worked in the field every day for 3 wk prior to her illness. She presented with the following clinical parameters: blood pressure, 100/60 mmHg; pulse rate, 84/min; respiratory rate, 20/min; and body temperature, 38.1 ℃. Hematochemical tests revealed the following: white blood cell count, 2.97/L (neutrophils 59%, lymphocytes 25%); hemoglobin, 12.4 g/dL; platelets, 117/L; erythrocyte sedimentation rate, 22 mm/h; C-reactive protein, 5.05 mg/dL; aspartate aminotransferase (AST)/alanine aminotransferase (ALT), 426/299 U/L; gamma-glutamyltransferase (γ-GTP)/alkaline phosphatase, 368/299 U/L; total bilirubin, 1.12 mg/dL; and lactate dehydrogenase/creatinine phosphokinase, 172/110 U/L (normal range, 180-460/26-20 U/L). The patient was negative for antibodies against acute hepatitis viruses A, B, D and E and for immunoglobulin M (IgM) against Epstein-Barr virus capsid antigen. PCR for cytomegalovirus and antigenemia tests were also negative. Antibodies involved in connective tissue diseases were absent. Specimens for immunohistochemical staining were obtained by ultrasound-guided needle aspiration to accurately evaluate the increased serum levels of liver enzymes. For immunohistochemical staining, we used a monoclonal anti-*O. tsutsugamushi* antibody that targets a recombinant 56 kDa protein of a clinical *O. tsutsugamushi* Boryong strain. Immunolocalization was performed using a polyvalent horseradish peroxidase polymer detection system according to the manufacturer's protocol (Lab Vision, Fremont, CA, United States)[5].

On the third day of hospitalization, a fever was evident (≥ 38 ℃), chills and headache persisted, and maculopapular rashes occurred. Thus, we conducted serological tests for scrub typhus, leptospirosis and hemorrhagic fever with renal syndrome (HFRS) as well as PCR for scrub typhus and leptospirosis. Hematoxylin and eosin staining of the biopsy specimens revealed mild inflammation with a small number of eosinophils and a dominance of lymphocytes in the portal areas. Piecemeal necrosis and interface hepatitis were not evident in the portal tract (Figure 1). Mild degrees of lobular disarray and ballooning degeneration of hepatocytes were observed in addition to the necrosis of isolated hepatocytes. However, there were no clusters of necrotic hepatocytes, bridging necrosis, intracanalicular bile plugs or intrahepatic cholestasis. Immunohistochemical staining using a monoclonal anti-*O. tsutsugamushi* antibody showed scattered positive immunoreactions in the cytoplasm of some hepatocytes (Figure 1D). Serological tests for *O. tsutsugamushi* performed on the third day of hospitalization revealed anti-*O. tsutsugamushi* IgM and immunoglobulin G (IgG) antibody titers of 1:40 and 1:1024, respectively. Nested PCR using the blood buffy coat was positive for the gene encoding the 56 kDa protein of *O. tsutsugamushi*. Serological tests were negative for HFRS and leptospirosis. Based on these findings, a comprehensive physical examination was conducted, which revealed an eschar on one of her labia minora. The patient reported that she had urinated during field work. Finally, she was definitively diagnosed with scrub typhus. A 5-d course of doxycycline improved the symptoms of fever, chills and headache and her unusually high levels of liver enzymes and low platelet count. She was discharged from the hospital without any complications. At the time of discharge, serological tests showed anti-*O. tsutsugamushi* IgM and IgG titers of 1:5120 and 1:16 384, respectively.

DISCUSSION

Fever, headache and skin rash are potential indicators of rickettsial diseases[6]. In the present case, the patient presented with fever, diarrhea and elevated serum levels of liver enzymes without a typical skin rash or eschar suggestive of scrub typhus. We initially considered infections caused by hepatitis virus, other infectious diseases such as tuberculosis and autoimmune hepatitis. Serological tests, serum protein electrophoresis and the use of antinuclear antibodies all yielded negative results, but immunohistochemical staining of a biopsy specimen revealed hepatocytes positive for *O. tsutsugamushi*. Because a maculopapular skin rash developed during hospitalization and because positive immunohistochemical staining was confirmed, a thorough history and a comprehensive physical examination were conducted, which confirmed an eschar on a labium minus and a history of urination during field work. These results reflect the importance of a thorough physical examination that includes the genitalia[7].

Although many studies have reported increases in liver enzymes in patients with scrub typhus[2], there have been only a few reports on the histopathological features of this disease[4]. In this case, the primary lesion was portal triad inflammation, which is consistent with the results of a previous study indicating that *Rickettsia* and *Orientia* species mainly cause such inflammation due to tropism for the vascular endothelium[8]. Rickettsioses cause focal lesions in various organs but do not cause extensive hepatic lesions that are sufficient to cause organ failure[9]. In addition, although Rocky Mountain Spotted Fever causes the focal death of some hepatocytes and increases in the serum levels of liver enzymes due to hepatic infection, hepatic failure does not occur[8]. In our case, immunohistochemical staining showed scattered focal positive immunoreactions. *O. tsutsugamushi* hepatitis is speculated to cause primarily intrahepatic sinusoidal endothelial vasculitis and to increase the serum levels of

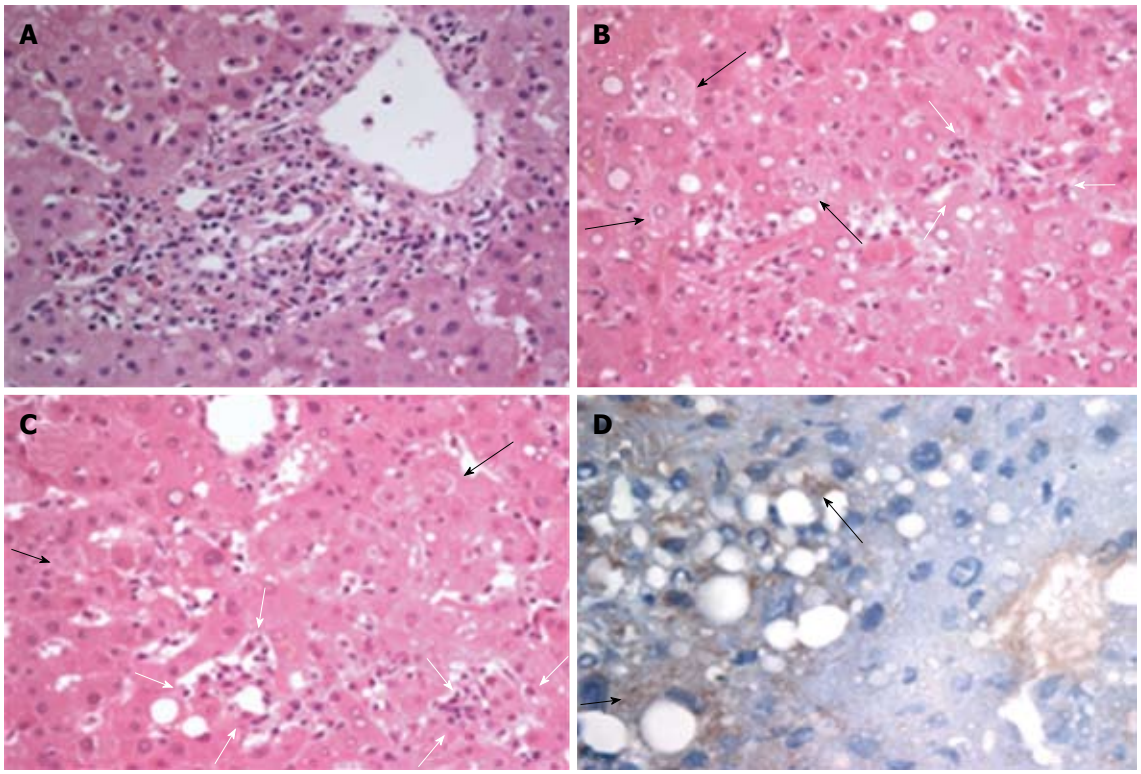


Figure 1 Histopathological analysis of a liver specimen from a patient with scrub typhus. A: Portal inflammation showing lymphocytes and some eosinophils devoid of distinct interface hepatitis; B, C: The degeneration of individual hepatocytes (black arrows), mild ballooning changes with lobular disarray and small clusters of mononuclear cell infiltration (white arrows) identified in the lobules. Neither cholestasis nor hepatocyte apoptosis are evident (hematoxylin and eosin stain, ×400 magnification); D: Immunohistochemical staining for *Orientia tsutsugamushi* demonstrating scattered positive immunoreactions (arrows) in the cytoplasm of the hepatocytes (labeled streptavidin-biotin method, counterstained by hematoxylin, ×400 magnification).

AST, ALT and γ-GTP due to direct cytopathic liver damage[10]. In contrast, host immune responses have been shown to cause viral hepatitis-induced liver damage[11,12]. The hepatitis virus multiplies within the cytoplasm of hepatocytes, causing non-cytopathic inflammation and hepatocellular damage as well as the destruction of infected hepatocytes through human leukocyte antigen-restricted, hepatitis A virus-specific CD8+ T lymphocytes and natural killer cells. Because hepatocyte degeneration, necrosis and apoptosis are more severe in acute viral hepatitis, it is conceivable that the serum levels of AST and ALT would be significantly higher in acute viral hepatitis than in scrub typhus hepatitis. Unlike patients with scrub typhus hepatitis, patients with viral hepatitis exhibit hepatic cholestasis, including intracanalicular bile plugs or the brown pigmentation of hepatocytes; this hepatic cholestasis can markedly increase the serum levels of γ-GTP and alkaline phosphatase[13,14]. Further studies are needed to confirm the relationship between the laboratory and histopathological findings.

Although granuloma formation in the liver is a characteristic finding in patients with scrub typhus, our patient did not have granulomatous hepatitis. It is thought that granulomatous hepatitis can be observed in some patients with scrub typhus.

In summary, this case has 2 clinical implications. First, our patient was diagnosed early with scrub typhus by immunohistochemistry using a monoclonal antibody for *O. tsutsugamushi*. The immunohistochemical analysis showed scattered focal positive immunoreactions in the cytoplasm of some hepatocytes. Second, our report supports previous findings demonstrating that scrub typhus hepatitis causes mild portal inflammation and lobular activities without causing significant interface hepatitis or intralobular hepatocyte death due to focal direct liver damage. In contrast, acute viral hepatitis secondary to host immune response-induced liver damage causes severe lobular disarray with diffuse hepatocyte degeneration, necrosis and apoptosis as well as findings of hepatic cholestasis, including intracanalicular bile plugs and the brown pigmentation of hepatocytes.

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CASE REPORT

Air in the main pancreatic duct: A case of innocent air

Yun Ji Kim, Hyung Keun Kim, Young Seok Cho, Sung Soo Kim, Hiun Suk Chae, Seung Kyong Kim, Eun Sun Kim, Su Yeon Lee

Yun Ji Kim, Hyung Keun Kim, Young Seok Cho, Sung Soo Kim, Hiun Suk Chae, Seung Kyong Kim, Eun Sun Kim, Su Yeon Lee, Division of Gastroenterology, Department of Internal Medicine, College of Medicine, Uijeongbu St. Mary's Hospital, The Catholic University of Korea, Uijeongbu 480717, South Korea

Author contributions: Kim YJ wrote the paper; Kim HK revised the paper; Cho YS, Kim SS, Chae HS, Kim SK, Kim ES and Lee SY provided medical care.

Correspondence to: Hyung Keun Kim, MD, PhD, Division of Gastroenterology, Department of Internal Medicine, College of Medicine, Uijeongbu St. Mary's Hospital, The Catholic University of Korea, 65-1 Geumo-dong, Uijeongbu 480717, South Korea. hykkim@catholic.ac.kr

Telephone: +82-31-8203016 Fax: +82-31-8472719
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Abstract

Air in the main pancreatic duct has been reported only rarely and might be associated with either a spontaneous or a surgically induced alteration of the anatomy of the biliary tract. We report a case of "innocent" air found incidentally in the main pancreatic duct. To our knowledge, this is only the third such case reported. A 54-year-old woman presented with hemoptysis that had lasted for 3 d. She underwent a chest computed tomography scan, which revealed not only focal bronchiectasis in the left lower lobe, but also air in the main pancreatic duct and dilatation of the common bile duct. She was managed conservatively for the hemoptysis and no further problems developed. She had no specific gastrointestinal symptoms and had no history of surgery or medication. Her laboratory parameters were normal. Magnetic resonance cholangiopancreatography also demonstrated air in the main pancreatic duct and a dilated common bile duct (CBD). Duodenoscopy revealed separate biliary and pancreatic orifices with patulous openings and some air bubbles appearing in the pancreatic orifice. Endoscopic retrograde cholangiopancreatography (ERCP) showed the dilated CBD and pancreatic duct with some air bubbles, but no other abnormal lesions. She was discharged with no further problems. Most patients with air in the main pancreatic duct have had a pancreatobiliary disease, or a history of pancreatobiliary disease, pancreatobiliary surgery or sphincterotomy. If the air is innocent, as in our case, ERCP should be performed to evaluate any altered sphincteric function or anatomy such as patulous openings.

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Key words: Air; Main pancreatic duct; Endoscopic retrograde cholangiopancreatography

Peer reviewer: Dr. Xiaoyun Liao, Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Room JF-208E, Boston, MA 02215, United States

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INTRODUCTION

Air in the pancreas is unusual and is usually related to an abscess, emphysematous pancreatitis or a pancreatic fistula[1,2]. There have been several cases reported, including one case series of air in the main pancreatic duct rather than in the pancreas itself[3-9].

This phenomenon has been reported in a child with biliary and pancreatic ascariasis with liver abscesses and a pancreatic pseudocyst[3], in an adult patient with cystic dilatation of the main pancreatic duct limited to the

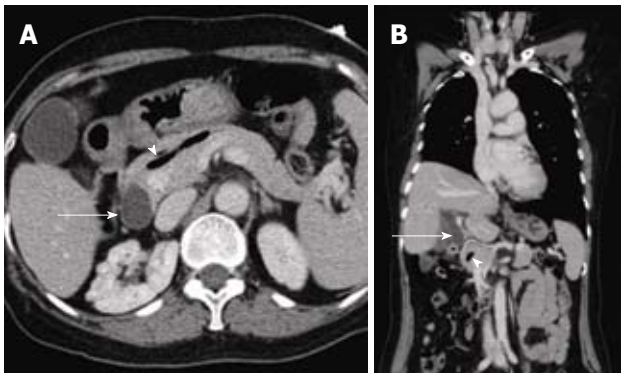


Figure 1 Computed tomography scans revealed air in the main pancreatic duct (arrowhead) and dilatation of the common bile duct (arrow). A: Axial scan; B: Coronal scan.



Figure 3 Endoscopic retrograde cholangiopancreatography showed dilatations of the common bile duct and pancreatic duct with some air bubbles (arrowheads), but no other abnormal lesions.

head of the organ[4], in a patient after transampullary septectomy[5], in 11 patients with various acute or chronic pancreatic and/or biliary diseases[6], in three infants with a congenital duodenal obstruction[7], in a middle-aged man with a history of acute pancreatitis[8], and in a young woman with acute pancreatitis and a pseudocyst[9]. Among these patients, most had a pancreatobiliary disease, or a history of pancreatobiliary disease, pancreatobiliary surgery or sphincterotomy, whereas only two patients had no such background.

We report a case of "innocent" air in the main pancreatic duct found incidentally. The patient had no history of a pancreatobiliary disease and had no pancreatic or biliary symptoms.

CASE REPORT

A 54-year-old woman presented at our hospital's division of pulmonology with hemoptysis that had lasted for 3 d. She underwent a chest computed tomography (CT) scan, which revealed not only focal bronchiectasis in the left lower lobe, but also air in the main pancreatic duct (arrowhead in Figure 1) and dilatation of the common bile duct (CBD; arrow in Figure 1). She was managed conservatively for the hemoptysis and no further problems developed. She was referred to the gastrointestinal (GI) division for an evaluation. She had no specific GI symptoms such as abdominal pain and had no history of surgery or medication. Her laboratory parameters were normal (hemoglobin, 14.8 g/dL; white blood cell, 6400/mm^3; aspartate aminotransferase/alanine aminotransferase, 17/16 IU/L; total bilirubin, 1.14 mg/dL; alkaline phosphatase, 182 IU/L; γ-glutamyl transpeptidase, 31 IU/L; amylase, 94 IU/L). To better evaluate the pancreatic ductal system, magnetic resonance cholangiopancreatography (MRCP) was carried out. This also demonstrated air in the main pancreatic duct and the dilated CBD. To further evaluate the ampulla of Vater and the pancreatobiliary ductal system, endoscopic retrograde cholangiopancreatography (ERCP) was carried out. A duodenoscopic examination revealed separate biliary (arrow in Figure 2) and pancreatic orifices with patulous openings and some air bubbles appearing in the pancreatic orifice (arrowhead in Figure 2). The ERCP showed the dilated CBD and pancreatic duct with some air bubbles (Figure 3), but no other abnormal lesions. She was discharged with no further problems.

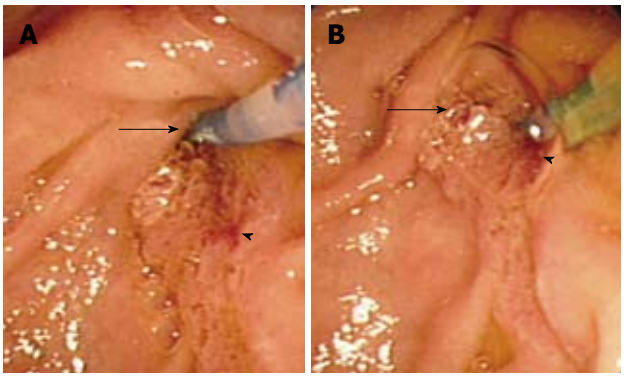


Figure 2 Duodenoscopy revealed separate biliary and pancreatic orifices. A: A catheter inserted into the biliary orifice (arrow); B: A catheter inserted into the pancreatic orifice (arrowhead). The pancreatic orifice was patulous with some air bubbles appearing in it.

DISCUSSION

Here, we report a case of "innocent" air found incidentally in the main pancreatic duct. The patient had no pancreatobiliary disease or history of it. She had separate biliary and pancreatic orifices with patulous openings but displayed no pancreatic or biliary symptoms. To our knowledge, this is only the third case reported of innocent air in the main pancreatic duct[4,6].

This phenomenon has been reported only rarely and might be associated with either a spontaneous or surgically induced alteration of the anatomy of the biliary tract. The origin of such air is not known, but there have been two possible explanations given[6,10]. First, it is likely that air in the pancreatic duct is the result of biliary-pancreatic reflux in patients who have received biliary-enteric anastomoses or have undergone sphincterotomy. Second, in patients who have not undergone

such operations, the likely cause of pancreatic duct air is a duodenal-pancreatic duct reflux. This possibility arose from the observation that six of 11 patients with air in the pancreatic duct had undergone previous biliary or pancreatic surgery or had undergone sphincterotomy; these patients all had pneumobilia[6]. However, these patients comprised only 5% of all who had received biliary anastomoses or had undergone sphincterotomy, so this mechanism seemed to operate only in a minority of such patients. Duodenal-pancreatic duct reflux occurring in patients who have not undergone pancreatobiliary surgery or sphincterotomy could be explained by the animal experiment of DiMagno *et al*[10]. That is, during duodenal phase Ⅲ motor activity of the interdigestive period, the mean duodenal pressure at times exceeds the mean pancreatic pressure, and reflux of duodenal contents into the pancreatic duct can occur. Generally, the sphincter of Oddi plays a major role in regulating secretory flow and preventing such reflux, but patients who have undergone a sphincterotomy or have had papillitis might have altered sphincter function so that reflux could occur more commonly. However, in three patients with a common channel, air was present only in the pancreatic duct and not in the biliary system[6]. This could be explained neither by a biliary-pancreatic reflux nor by a duodenal-pancreatic duct reflux. Further investigations on the presence of air in the pancreatic duct are needed.

In all previous reports, air in the pancreatic duct was demonstrated by CT scans[3,4,8,9] or ultrasonography[5-7]. CT is known to be sensitive for detecting small amounts of gas. Generally, air in the pancreatic duct is never demonstrated on plain radiographs. In our case, air in the pancreatic duct was found first by a CT scan and then found in MRCP and ERCP studies. In a previous case series, nine of 11 patients underwent a second ultrasound examination and air in the pancreatic duct was only confirmed in five[6]. In another case, air in the pancreatic duct found first on CT scans was not shown in the second MRCP[8]. This spontaneous disappearance of air seemed to be related to the cyclically variable diameter of the pancreatic duct associated with air expulsion or the phasic motor activity of the sphincter of Oddi[6]. As in our case, why air in the pancreatic duct persisted was not understood fully. However, in our case, the major duodenal papilla had separate biliary and pancreatic orifices with patulous openings. This anatomy could have been one cause of air in the pancreatic duct that also affected the persistence of air. Why there was no pneumobilia and the CBD was dilated is also not understood fully; possibly the degree of altered sphincteric function differed between the separate biliary and pancreatic orifices.

In most cases reported in the literature, the patients had a history of biliary and/or pancreatic disease, such as pancreatitis or cholangitis, or a history of pancreatobiliary surgery or sphincterotomy[3,5-9]. Two patients had no history of pancreatobiliary disease or surgery and no pancreatobiliary symptoms[4,6]. Our patient also had no history of pancreatobiliary disease or surgery and no related symptoms. Therefore, this appears to be the third reported case of innocent air in the pancreatic duct. Separate duct orifices with patulous openings might be related to the presence of air in the pancreatic duct and the dilated CBD, but how such patulous openings formed is not known.

In summary, when air in the pancreatic duct is discovered, the physician should investigate a history of pancreatobiliary disease, a history of pancreatobiliary surgery and sphincterotomy and any related symptoms. If the air is innocent, as in our case, ERCP should be performed to evaluate any altered sphincteric function or anatomy such as patulous openings.

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Dr. Gustav Paumgartner, Professor, Klinikum Grosshadern, Marchioninistr. 15, 81377 Munich, Germany

Carlos J Pirola, PhD, FAHA, Medical Research Institute A Lanari, Combatientes de Malvinas 3150, Buenos Aires 1427, Argentina

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January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
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January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
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February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
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March 30-April 2, 2012
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27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
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May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

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PancreasFest 2012
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2012 Joint International Neurogastroenterology and Motility Meeting
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Kiev, Ukraine

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November 9-13, 2012
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December 1-4, 2012
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Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

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In press

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Organization as author

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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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EDITORIAL

Cell sheet technology for regeneration of esophageal mucosa

Ryo Takagi, Masayuki Yamato, Nobuo Kanai, Daisuke Murakami, Makoto Kondo, Takaaki Ishii, Takeshi Ohki, Hideo Namiki, Masakazu Yamamoto, Teruo Okano

Ryo Takagi, Masayuki Yamato, Nobuo Kanai, Daisuke Murakami, Makoto Kondo, Takaaki Ishii, Takeshi Ohki, Teruo Okano, Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo 162-8666, Japan
Nobuo Kanai, Takeshi Ohki, Masakazu Yamamoto, Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Tokyo 162-8666, Japan
Daisuke Murakami, Makoto Kondo, Takaaki Ishii, Hideo Namiki, Graduate School of Science and Engineering, Waseda University, Tokyo 162-8480, Japan
Author contributions: Yamato M, Ohki T, Namiki H, Yamamoto M and Okano T designed this research; Takagi R, Kanai N, Murakami D, Kondo M and Ishii T fabricated cell sheets; Kanai N and Ohki T performed endoscopic surgery; and Takagi R and Yamato M wrote this manuscript.
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Correspondence to: Teruo Okano, PhD, Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, TWIns, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan. tokano@abmes.twmu.ac.jp
Telephone: +81-3-53679945 Fax: +81-3-33596046
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Abstract

The progress of tissue-engineering technology has realized development of new therapies to treat various disorders by using cultured cells. Cell- and tissue-based therapies have been successfully applied to human patients, and several tissue-engineered products have been approved by the regulatory agencies and are commercially available. In the review article, we describe our experience of development and clinical application of cell sheet-based regenerative medicine. Endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) have been shown to be useful for removal of gastrointestinal neoplasms with less invasiveness compared with open surgery, especially in esophageal surgery. However, postoperative inflammation and stenosis are major complications observed after intensive mucosal resection. Therefore, we have developed novel regenerative medicine to prevent such complications and promote wound healing of esophageal mucosa after EMR or ESD. Transplantable oral mucosal epithelial cell sheets were fabricated from patients' own oral mucosa. Immediately after EMR or ESD, fabricated autologous cell sheets were endoscopically transplanted to the ulcer sites. We performed a preclinical study with a canine model. In human clinical settings, cell culture and cell sheet fabrication were performed in clean rooms according to good manufacturing practice guidelines, and pharmaceutical drugs were used as supplements to culture medium in place of research regents used in animal study. We believe that cell-based regenerative medicine would be useful to improve quality of life of patients after EMR or ESD.

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Key words: Cell sheet; Endoscopic resection; Esophageal stenosis; Oral mucosa; Good manufacturing practice

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INTRODUCTION

Endoscopic mucosal resection (EMR) is one of the standard techniques for removing superficial early-stage cancers in the gastrointestinal tract with low invasiveness compared with open surgery[1,2]. In the case of the esophagus, EMR is a useful operation for removing m1 (epithelial cancers restricted to the intraepithelial layer) and m2 (mucosal cancers with invasion of intermediate depth) carcinomas without esophageal reconstruction. To permit large *en bloc* resection of cancerous lesions without technical restriction of EMR, endoscopic submucosal dissection (ESD) was developed as a new mucosal resection technique with development of endoscopic devices[3-5]. Early-stage esophageal cancers are removed using a hook-knife, which is an endoscopic device used to perform ESD[6]. ESD is a more impressive operative procedure than EMR for reducing the recurrence of esophageal squamous cell carcinoma[7]. Although these developments of endoscopic surgery contribute low invasive cancer resection for patients suffering from esophageal cancer, there are some postoperative complications after ESD. Esophageal stenosis is a major complication caused by endoscopic resection, and the stenosis is significantly associated with the mucosal defect involving over three-fourths circumference of the esophagus lumen[8]. The esophageal stenosis caused by aggressive ESD considerably affects the patient's quality of life, since the patient has to receive treatment with balloon dilation or temporary stents to expand the esophageal stricture with further inflammation and postoperative pain. These physical dilations carry a risk of perforation[9]. Treatment with anti-inflammatory drugs after endoscopic resection may be an effective therapy for preventing stricture after ESD[10,11].

With recent progression of tissue engineering and regenerative medicine, there are some reports proposing new technologies using biological scaffolds[12] or cell suspensions[13,14] for preventing the esophageal stenosis caused by mucosal defects. We have developed a novel method of endoscopic transplantation of autologous epithelial cell sheets immediately after ESD to prevent the postoperative complications[15]. Transplantable tissue-like epithelial cell grafts are fabricated by cell sheet technology. On the basis of results obtained with canine and porcine models, we have used this technology with human patients since 2008.

CELL SHEET TECHNOLOGY FOR REGENERATION OF ESOPHAGEAL MUCOSA

Tissue engineering by using cell sheet technology

The concept of tissue engineering was originally proposed by Langer *et al*[16]. Conventionally, biodegradable polymer scaffolds have been used to reconstruct tissue architecture, and cells are seeded on them. The technique should be useful to reconstruct bone and cartilage, having a large amount of extracellular matrices (ECM) and few cells. However, scaffold-based tissue engineering would not be optimal for the regeneration of parenchymal tissues filled with a huge amount of cells and faint ECM. Therefore, we have proposed an alternative method of tissue reconstruction by using transplantable cell sheets to eliminate biodegradable scaffolds.

In order to fabricate transplantable cell sheets without any scaffolds, we employ temperature-responsive culture surfaces, onto which poly (N-isopropylacrylamide) is covalently immobilized to control cell adhesion/detachment with a simple temperature change[17]. Cells adhere, spread, and proliferate on temperature-responsive surfaces at 37 ℃, which is the normal temperature for mammalian cell culture. By reducing temperature below 32 ℃, cells spontaneously detach from the surfaces without proteolytic enzyme such as trypsin, since the grafted polymer becomes hydrophilic. When the temperature is reduced after cells reach confluence, all the cells are harvested as a single contiguous cell sheet. Because this technique eliminates trypsin for cell harvest, all the cell membrane proteins including growth factor receptors, ion channels and cell-to-cell junction proteins are intact even after cell and cell sheet harvest. Furthermore, ECM deposited during cell culture is retained under cell sheets[18]. Therefore, cell sheets easily integrate to transplanted sites. With this technique, many types of epithelial cell sheets are fabricated and subjected to regenerative medicine of skin[19], cornea[20], urinary bladder[21], and trachea[22].

Cultured epithelial cells for clinical application

Cultured autologous epidermal keratinocytes have been used as cell grafts to treat burns as the first therapy using cultured cells[23]. A transplantable epidermal cell graft is fabricated using murine 3T3 feeder layer cells[24]. Human keratinocytes co-cultured with 3T3 feeder cells proliferate and show stratification as *in vivo* with characteristics of native epithelial tissues including tonofilaments and desmosomes. Interestingly, human epidermal keratinocytes can be serially cultured to more than 150th passages *in vitro*[25]. The cultivation of human keratinocytes achieved clinical treatments for severe burns[26] and giant congenital nevi[27] by transplantation of cultured autologous keratinocyte grafts. Moreover, allogeneic keratinocyte grafts are also used for skin ulcers[28]. In these cases, keratinocyte sheets are harvested with bacteria-derived dispase treatment.

Oral mucosal epithelium is also stratified squamous epithelium, and on the basis of the feeder layer method, the epithelial cells have been used as a cell source to fabricate transplantable epithelial cell grafts for treating oral mucosa[29-31]. Moreover, since extraction of oral mucosal tissue is easy, cultured human oral mucosal epithelial cells are selected as useful cell grafts for ectopic transplantation, such as skin[30,32]. We have successfully applied cultured autologous oral mucosal epithelial cell sheets fabricated on temperature-responsive culture inserts to treat human patient cornea suffering from limbal epi-

thelial stem cell deficiency[33,34]. Furthermore, we utilized cultured autologous oral mucosal epithelial cell sheets for bladder reconstruction by the transplantation onto a demucosalized gastric flap in a canine model[35]. We believe that cultured oral mucosal epithelial cell sheets fabricated on temperature-responsive surfaces are useful for treatment of many types of epithelial diseases as well as reconstruction of defective tissues.

Fabrication of transplantable oral mucosal epithelial cell sheets in the clinical setting

To promote healing of esophageal ulcers after ESD for the prevention of postoperative complications, we performed endoscopic transplantation of cultured autologous oral mucosal epithelial cell sheets in the clinical setting. From the safety view point, animal-derived materials, such as bovine serum and 3T3 feeder cells should be eliminated as much as possible. For example, human embryonic stem cells cultured under a typical culture condition using animal-derived serum replacements and mouse feeder layer express an immunogenic nonhuman sialic acid[36]. Moreover, cells co-cultured with a mouse feeder layer are classified as xenogeneic products by United States Food and Drug Administration.

We reported that oral mucosal epithelial cells make transplantable stratified cell sheets on temperature-responsive cell culture inserts having micropores to supply culture medium from the basal cell surfaces even without a 3T3 feeder layer and allogeneic serum[37]. The method can, in principle, permit the cultured human oral mucosal epithelial cell sheets to be used as an autologous epithelial cell sheet graft for human patients[38]. Therefore, the autologous epithelial cell sheets are not, in principle, rejected after transplantation onto the esophageal ulcer and may be replaced by native epithelium in the esophagus. Moreover, the culture medium for the preparation of cultured oral mucosal epithelial cell sheets is modified to exclude laboratory reagents as much as possible for the clinical use[39].

Transplantable epithelial cell sheets are fabricated in a cell-processing center (CPC) with clean rooms according to the good manufacturing practice guideline in the clinical study. The cleanliness of the CPC is controlled by air conditioning systems using high efficiency particulate air filters, and standard operation procedures (SOP) are documented to keep a sterile environment of cell culture rooms in the CPC. The environment of the CPC is continuously monitored with various monitors including aerosol particles, temperature, and humidity, and various sterilization tests are performed to validate the cleanliness of the environment. In these tests culture rooms in the CPC were successfully kept sterile[40,41]. SOP of the culture method was also documented for the prevention of human error of operators to fabricate cell sheets highly reproducibly. Consequently, transplantable human epithelial cell sheets were successfully fabricated in a clinical study to treat ten human patients, and validation tests to demonstrate the quality assurance were performed one day before the ESD and transplantation.

From the average yield of the cells isolated from excised human oral mucosal tissues of healthy volunteer donors, we concluded that approximately 0.3 cm^2 of oral mucosal tissue is needed to fabricate one transplantable epithelial cell sheet having an area of 2.8 cm^2 (Figure 1A and B). The degree of cell stratification is an important point to enable reproducible cell sheet harvesting from temperature-responsive culture inserts. Figure 1C and D show the growth of seeded human oral mucosal epithelial cells. These cells became confluent on the surface of temperature-responsive culture inserts, and elevation of cell density accompanied with cell stratification was observed after a further 5 d.

Endoscopic transplantation of epithelial cell sheet onto esophageal ulcer immediately after ESD

Since 2008, we have performed a clinical study using cultured autologous human oral mucosal epithelial cell sheets to treat esophageal ulcer after endoscopic resection of a mucosal neoplasm. Endoscopic transplantation of the cultured epithelial cell sheets is performed using a support membrane to grasp by endoscopic forceps[42]. Epithelial cell sheets transplanted onto esophageal ulcer with a support membrane in a swine model are shown in Figure 2. One epithelial cell sheet fabricated on temperature-responsive culture insert (23 mm in diameter) covers the ulcer surface involving approximately one-third circumference of the esophagus lumen (Figure 2A and B), and three or four epithelial cell sheets were needed to cover the full circumferential ulcer (Figure 2C and D).

Although more research is needed for understanding the mechanism and correlation between ulcer size and number of transplanted epithelial cell sheets for preventing inflammation and postoperative stenosis, serial cultivation is a useful method of preparation of epithelial cells from small tissue for fabricating cell grafts to transplant onto a large ulcer after aggressive ESD. Serial culture using low calcium concentration medium (LCM) is a useful method to amplify a cell number of stratified squamous epithelial cells without using 3T3 feeder cells[43,44]. Interestingly, although normal human epidermal keratinocytes cultured in LCM show some mesenchymal-like phenotype, the keratinocytes can express the differentiation marker and stratify in differentiation-inducing culture conditions[45]. Since the conventional culture medium for serial cultivation of epithelial cells typically includes animal-derived material, the medium is not useful for clinical use. The modification of the LCM would be important for fabricating epithelial cell sheets to transplant onto large esophageal ulcers after aggressive ESD without any complications.

CONCLUSION

This article introduces cell sheet technology to fabricate stratified epithelial cell sheet grafts for preventing postoperative inflammation and stenosis after endoscopic re-

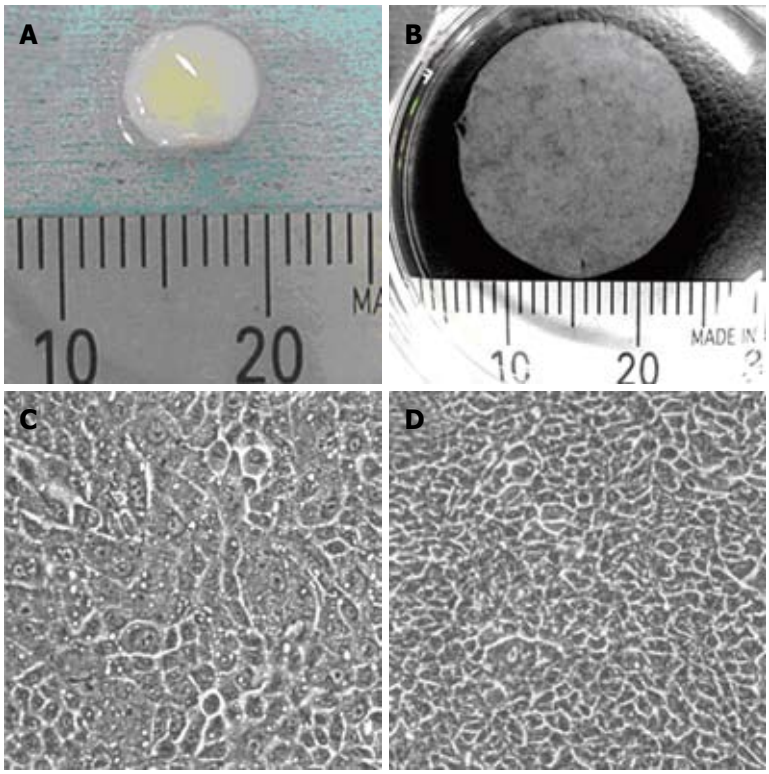


Figure 1 Transplantable oral mucosal epithelial cell sheet. A: Swine buccal oral mucosal tissue taken by punch biopsy; B: Human oral mucosal epithelial cell sheet harvested from temperature-responsive cell culture insert; C: Cultured human oral mucosal epithelial cells just become confluent on the surface of temperature responsive culture insert; D: The oral mucosal epithelial cells cultured for more than 5 d.

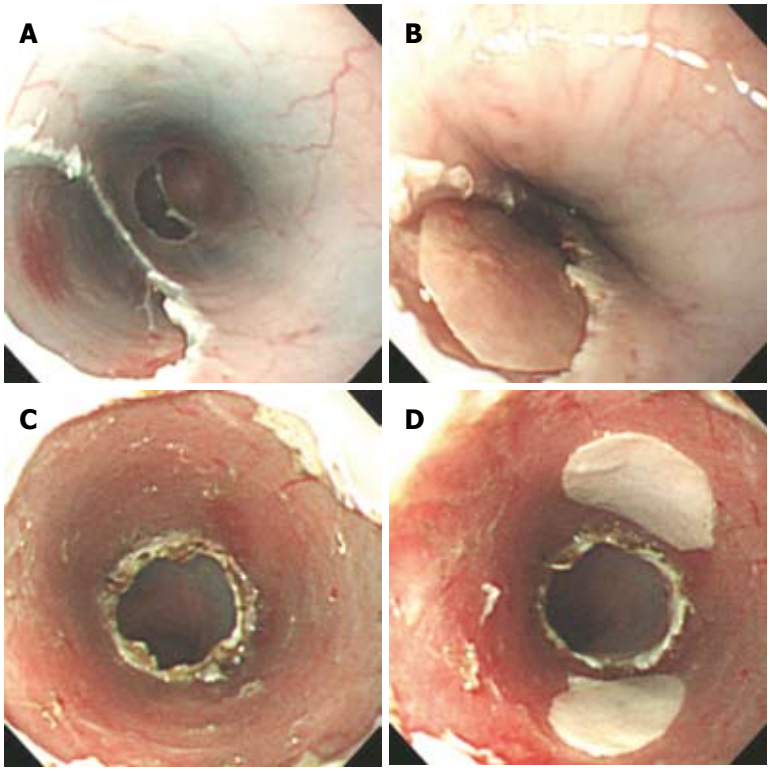


Figure 2 Transplantation of oral mucosal epithelial cell sheets on esophageal ulcer surface in animal model. A: Swine esophageal ulcer after endoscopic mucosal resection (EMR); B: Swine oral mucosal epithelial cell sheet transplanted on esophageal ulcer after EMR; C: Swine esophageal ulcer after endoscopic submucosal dissection (ESD); D: Two swine oral mucosal epithelial cell sheets transplanted on esophageal ulcer after ESD.

section for removing early-stage cancers from the esophagus. The efficacy of the oral mucosal epithelial cell sheet has been demonstrated, and the method for fabricating the cell sheet has also developed to use the cell graft as a clinical application for treating esophageal ulcers. This new therapy has been developed by integration of endoscopic technology and cell sheet technology. We believe that cell sheet technology will meet new technology and then the integration will create new therapies for treating patients without any specific therapies.

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GUIDELINES FOR CLINICAL PRACTICE

Irritable bowel syndrome: Diagnosis and pathogenesis

Magdy El-Salhy

Magdy El-Salhy, Section for Gastroenterology, Department of Medicine, Stord Helse-Fonna Hospital, 5416 Stord, Norway
Magdy El-Salhy, Section for Gastroenterology, Institute of Medicine, University of Bergen, 5029 Bergen, Norway
Supported by Grants from Helse-Fonna
Correspondence to: Magdy El-Salhy, Professor, Consultant Gastroenterologist, Section for Gastroenterology, Department of Medicine, Stord Helse-Fonna Hospital, Tysevegen 64, 5416 Stord, Norway. magdy.el-salhy@helse-fonna.no
Telephone: +47-5-3491000 Fax: +47-5-3491001
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Abstract

Irritable bowel syndrome (IBS) is a common gastrointestinal (GI) disorder that considerably reduces the quality of life. It further represents an economic burden on society due to the high consumption of healthcare resources and the non-productivity of IBS patients. The diagnosis of IBS is based on symptom assessment and the Rome Ⅲ criteria. A combination of the Rome Ⅲ criteria, a physical examination, blood tests, gastroscopy and colonoscopy with biopsies is believed to be necessary for diagnosis. Duodenal chromogranin A cell density is a promising biomarker for the diagnosis of IBS. The pathogenesis of IBS seems to be multifactorial, with the following factors playing a central role in the pathogenesis of IBS: heritability and genetics, dietary/intestinal microbiota, low-grade inflammation, and disturbances in the neuroendocrine system (NES) of the gut. One hypothesis proposes that the cause of IBS is an altered NES, which would cause abnormal GI motility, secretions and sensation. All of these abnormalities are characteristic of IBS. Alterations in the NES could be the result of one or more of the following: genetic factors, dietary intake, intestinal flora, or low-grade inflammation. Post-infectious IBS (PI-IBS) and inflammatory bowel disease-associated IBS (IBD-IBS) represent a considerable subset of IBS cases. Patients with PI- and IBD-IBS exhibit low-grade mucosal inflammation, as well as abnormalities in the NES of the gut.

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Key words: Cholecystokinin; Chromogranin A; Diagnosis; Diet; Endocrine cells; Intestinal flora; Hereditary; Low-grade inflammation; Peptide YY; Serotonin

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INTRODUCTION

Irritable bowel syndrome (IBS) affects as many as 5%-20% of individuals worldwide (Figure 1)[1-31]. The annual incidence of IBS is between 196 and 260 per 100 000[32,33], with IBS occurring more often in women than in men, and being more commonly diagnosed in patients younger than 50 years of age[14,34-44]. IBS symptoms range from diarrhoea to constipation, or a combination of the two, with abdominal pain or discomfort existing alongside abdominal distension[45]. The degree of symptoms varies in different patients from tolerable to severe, and the time pattern and discomfort varies immensely from patient to patient[14,34-44]. Some patients complain of daily symptoms, while others report intermittent symptoms at intervals of weeks or months. IBS is not known to be associated with the development of serious disease or with excess mortality[46,47]. However, IBS causes a reduced quality of life with the same degree of impairment as major chronic diseases, such as diabetes, congestive heart failure, renal insufficiency and hepatic cirrhosis[48-50]. Although a minority (10%-50%) of IBS patients seek healthcare, they generate a substantial workload in both primary and secondary care[51-53]. The annual costs in the United States, both direct and indirect,

for the management of patients with IBS are estimated at 15-30 billion USD[37,54,55].

The treatment options for IBS have included pharmacological symptomatic relief of symptoms such as pain, diarrhoea or constipation. Evidence of the long-term benefit of pharmacological agents has been sparse, and new agents that have proven to be effective have raised issues concerning safety[56,57]. Alternative therapies, such as cognitive behavioural therapy and gut-directed hypnotherapy, have been used with good results[58]. Other non-pharmacological approaches have been also tried with proven effects on symptoms and the quality of life in patients with IBS[58].

The present review is an attempt to give an update on the diagnosis and pathogenesis of IBS, and to discuss some controversial issues in both the diagnosis and pathogenesis of IBS.

DIAGNOSIS

There is currently no biochemical, histopathological or radiological diagnostic test for IBS, with the diagnosis of IBS being based mainly on symptom assessment. Over the last few years, Rome working parties have generated detailed, accurate, and clinically useful definitions of the syndrome. As a result, the Rome criteria (Ⅰ, Ⅱ and Ⅲ) have been established (Table 1)[59,60]. In addition to these criteria, warning symptoms or red flags, such as age over 50 years, a short history of symptoms, nocturnal symptoms, weight loss, rectal bleeding, anaemia, and the presence of markers for inflammation or infections, should be excluded. IBS patients are sub-grouped on the basis of differences in the predominant bowel pattern as diarrhoea-predominant (IBS-D), constipation-predominant (IBS-C), or a mixture of both diarrhoea and constipation (IBS-M), and un-subtyped IBS in patients with an insufficient abnormality of stool consistency to meet the criteria for IBS C, D or M (Table 2). It has been reported that around one third of patients have IBS-D, one third have IBS-C, and the remainder have IBS-M[61-63]. The division of IBS patients into subtypes is useful for clinical practice and symptomatic treatment, but it is common for IBS patients to switch from one subtype to another over time. These patients are known as "alternators". More than 75% of IBS patients change to either of the other 2 subtypes at least once over a 1-year period[63].

The majority of gastroenterologists believe that a symptom-based diagnosis, such as that based on the Rome Ⅲ criteria, without red flags is enough for the diagnosis of IBS and that no further investigations are needed. The use of red flags in combination with Rome criteria has been found to be highly specific, but not particularly sensitive[64]. The American College of Gastroenterology Task Force does not recommend routine colonoscopy in patients younger than 50 years of age without any associated alarming symptoms[65]. The guidelines of the of the British Society of Gastroenterology go further, however, by recommending an examination of the colon earlier if there is a first degree relative affected by colorectal cancer who is younger than 45 years, or two first degree relatives of any age[66]. The British Society Of Gastroenterology also recommended further investigations in IBS-D due to the overlap with other diarrhoea diseases, such as coeliac and inflammatory bowel disease (IBDs)[66]. These recommendations seem to be suitable for detecting and diagnosing colorectal cancer in this group of patients, but not in other organic gastrointestinal (GI) diseases. It is rather difficult to clinically distinguish IBS from adult-onset coeliac disease (CD)[67-73], as the breadth of the spectrum of symptoms associated with IBS results in a potential for overlap of IBS and CD symptomatologies. The situation is further complicated by the fact that the abdominal symptoms of both IBS and CD patients are triggered by the ingestion of wheat products. In CD patients, this is due to a gluten allergy, while in IBS the effect is attributed to the long sugar polymer fructan in the wheat[74]. The prevalence of CD in IBS varies in different studies and varies from 0.04% to 4.7%[72,73,75-84]. Regardless of the number of CD patients among patients diagnosed with IBS, I believe that IBS patients from all subtypes should be routinely screened for CD, which is in line with current opinions in the field[84-86]. Distinguishing IBD from IBS, especially with mild disease activity, can be difficult[87]. Furthermore,

Table 1 Rome Ⅲ criteria for the diagnosis of irritable bowel syndrome[1]

Recurrent abdominal pain or discomfort with onset at least 6 mo prior to diagnosis, associated with 2 or more of the following, at least 3 d/mo in the last 3 mo
Improvement with defecation
Onset associated with change in frequency of stool
Onset associated with change in form (appearance) of stool
Symptoms that cumulatively support the diagnosis are:
Abnormal stool frequency (greater than 3 bowel movements per day or less than 3 bowels movements per week)
Abnormal stool form (lump/hard or loose/watery stool)
Abnormal stool passage (straining, urgency or feeling of incomplete evacuation)
Passage of mucous
Bloating or feeling of abdominal distension

[1]Adapted from reference [1] with the permission from Nova Science Publisher, Inc.

Table 2 Subtyping of irritable bowel syndrome[1]

IBS with constipation-hard or lumpy stools > 25% and loos or watery stools < 25% of bowel movements
IBS with diarrhea-loos or watery stools > 25% and hard or lumpy stools stools < 25% of bowel movements
Mixed IBS-loos or watery stools > 25% and hard or lumpy stools stools > 25% of bowel movements
Unsubtyped IBS-insufficient abnormality of stool consistency to meet criteria for IBS-C,D or M

[1]Adapted from reference [1] with the permission from Nova Science Publisher, Inc. IBS: Irritable bowel syndrome; IBS-C: IBS with constipation; IBS-D: IBS with diarrhea-loos; IBS-M: IBS with a mixture of both diarrhoea and constipation.

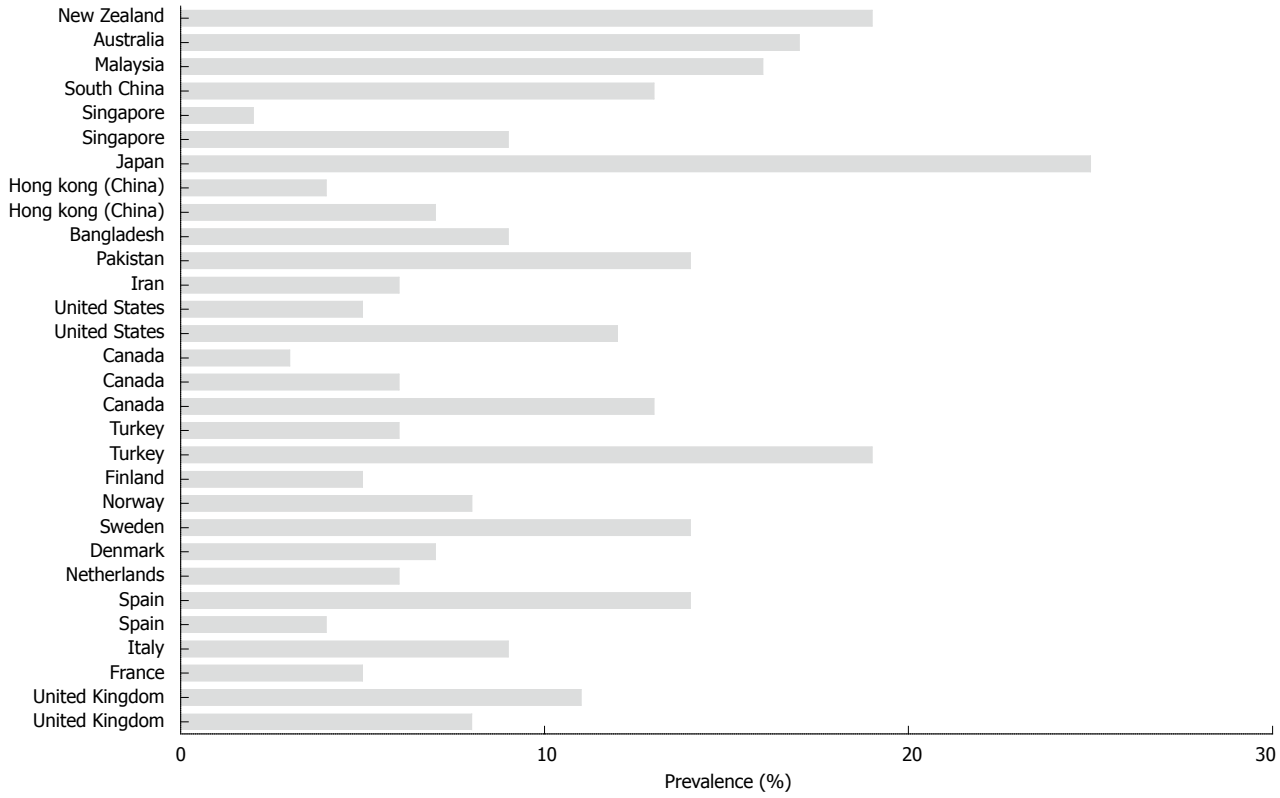


Figure 1 The prevalence of irritable bowel syndrome according to Rome criteria in different countries. Reproduced from reference [1] with permission from Nova Science Publisher, Inc.

IBS-like symptoms are frequently reported before the diagnosis of IBD[87-90]. Microscopic colitis (MC) and IBS have similar symptoms and a normal endoscopic appearance[91-101], and the diagnostic overlap between IBS, IBD and MC is important because of a potentially different treatment for each disorder. The prevalence of IBD in patients that fulfilled the Rome criteria without alarming symptoms varies between 0.4% and 1.9%[96-100], and MC from 0.7% to 1.5%[90-97]. It is conceivable, therefore, to conclude that symptom-based diagnosis of IBS may lead to a number of other GI disorders that require quite different management than IBS being missed. Sigmoidoscopy in IBS patients might be insufficient, however, as a considerable number of MC patients may not be identified without mucosal biopsies from the right colon[101]. Moreover, performing a sigmoidoscopy would not exclude Crohn's disease lesions in the terminal ileum, making ileocolonoscopy prefered, especially in IBS-D patients. This seems, at first sight, to add more economic burden to healthcare, which is already suffering from a lack of resources. IBS patients are already consuming a large amount of healthcare resources. However, performing an ileocolonoscopy would reassure IBS patients and prevent them from seeking a new examination, which would not increase the economic burden of this patient group on society, but instead use the existing resources effectively.

Several biomarkers for the diagnosis of IBS have been considered, but only gut transit measured by radio-isotope markers meets the criteria for reproducibility and availability[102]. However, radio-isotope tests themselves are expensive and of limited availability[102]. It has been reported that the chromogranin A-containing cell density is low in the duodenum of IBS patients (Figure 2)[103,104]. As chromogranin A is a general marker for endocrine cells[105,106], this finding indicates a general reduction in small intestinal endocrine cells in these patients. It has been proposed that the quantification of duodenal chromogranin A cell density could be used as a histopathological marker for the diagnosis of IBS[103,104]. Receiver-operator characteristic curves for chromogranin A cell density in the duodenum is given in Figure 3. The sensitivity and specificity at the cut-off < 31 cells/mm^2 in the duodenum are 91% and 89%, respectively. Screening of IBS patients for CD is now widely accepted. Thus, gastroscopy with duodenal biopsies can be used for excluding or confirming CD instead of blood tests, and the same biopsies can be used for the diagnosis of IBS. The duodenal endocrine cell types affected and their role in the pathogenesis of IBS is discussed in the next section.

PATHOGENESIS

Patients with IBS typically present with GI complaints for which physicians can find no organic cause. It is natural and understandable to make comparisons with hysteria, which is also predominant in women. Hysteria has been replaced in modern psychiatry by somatisation disorders and conversion disorders. The notion that IBS is a psychiatric disorder is deeply rooted in clinical practice.

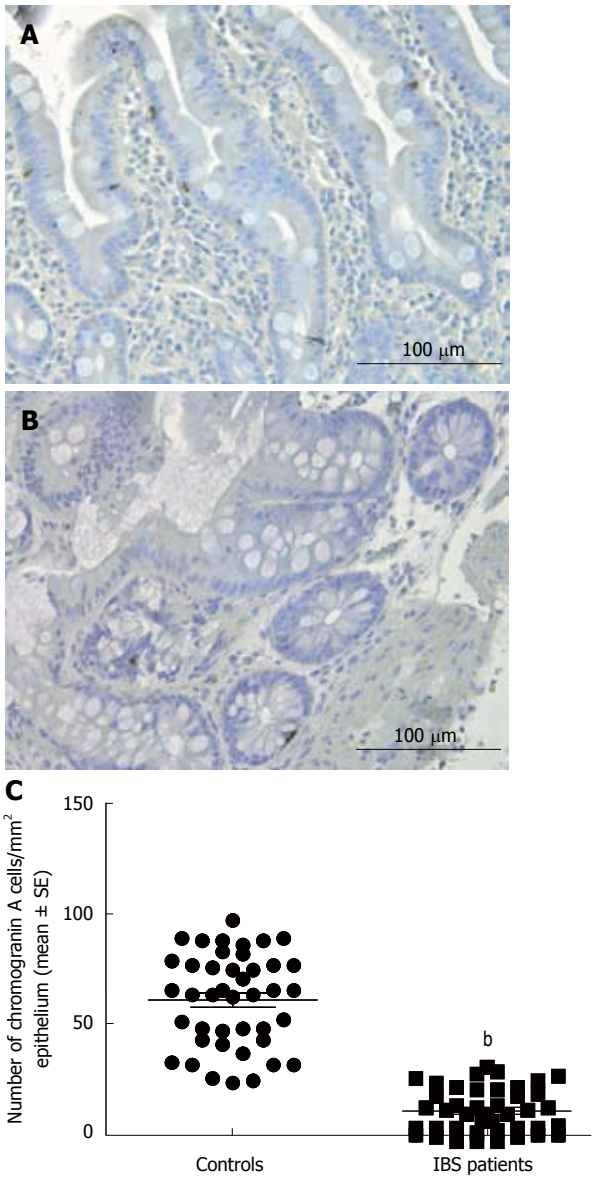


Figure 2 Chromogranin A cells in the duodenum. A: A healthy subject; B: A patient with irritable bowel syndrome (IBS); C: Controls and IBS patients. Reproduced from reference [1] with permission from Nova Science Publisher, Inc. $^{b}P < 0.01$ *vs* control group.

This situation was not improved by the huge number of publications on a selected group of IBS patients, which show that IBS patients are more likely to be psychiatrically ill and sexually or physically abused than the general population[107-121]. Many patients with IBS ignore their symptoms and regard them as a normal part of everyday life. IBS patients with anxiety, depression, somatisation or hypochondria are more liable to seek healthcare than other IBS patients. Unless this is borne in mind, incorrect conclusions can be drawn. A hospital-based case-control study showed that patients with IBS have a comparable health-related quality of life, level of psychological distress and occurrence of recent stressful life events to age-matched IBD patients[122]. These findings are interesting as IBD patients receive effective treatment and are treated with sympathy, understanding and support by their doctors as well as society. In contrast, IBS patients are offered non-effective treatments, are treated with mistrust and neglect by their doctors, feel that they are labelled as hypochondriacs and believe that they receive no support from society. It could be expected that IBS patients would be more anxious and depressed than IBD patients, but this is not the case. Two percent of patients diagnosed with IBS among the adult residents of Olmsted County, Minnesota, United States, were found to suffer from depression compared to the 16.2% incidence of depression in the entire population of the United States[122,123]. In conclusion, there is no convincing evidence to show that psychological factors play a role in the onset and/or progression of IBS[66].

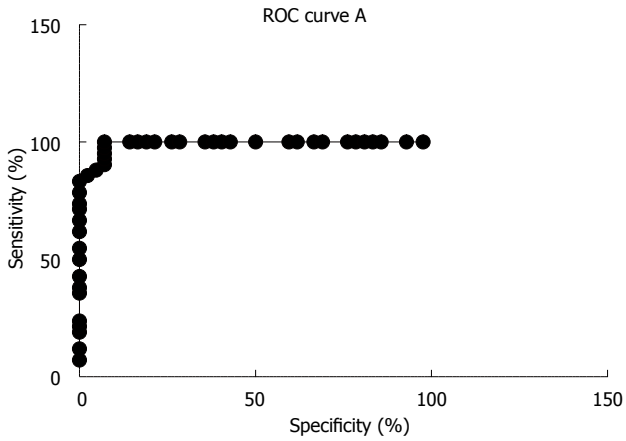


Figure 3 Receiver-operator characteristic for chromogranin A cell density in the duodenum. Reproduced from reference [1] with permission from Nova Science Publisher, Inc. ROC: Receiver-operator characteristic.

The pathogenesis of IBS appears to be multifactorial. There is evidence to show that the following factors play a central role in the pathogenesis of IBS: heritability and genetics, environment and social learning, dietary or intestinal microbiota, low-grade inflammation and disturbances in the neuroendocrine system (NES) of the gut.

Heritability and genetics

Up to 33% of patients with IBS had a family history of IBS compared to 2% of the controls[124]. In a study of a family cluster from Olmsted County, United States, a significant association was reported between having a first degree family member with bowel symptoms and presenting with IBS. In contrast, those who reported having a spouse with bowel symptoms were no more likely to present with IBS than the general population[125]. It was further shown that the prevalence of IBS was 17% in the relatives of patients compared to 7% in the relatives of spouses[126]. Another study showed that patients with IBS were more likely to present a family history of IBS than controls (33.9% and 12.6%, respectively). Moreover, 21.1% of IBS non-consulter patients reported a family history of IBS, in comparison with 12.6% of the control subjects[127].

In twin studies, a higher rate of IBS was reported in monozygotic twins than in dizygotic twins (33.3% *vs*

13.3%). Moreover, 56.9% of the variance was attributed to additive genetic factors, indicating a substantial genetic component in IBS[128-132]. In contrast, a study performed on British twin pairs did not show any significance in the rates of IBS between monozygotic and dizygotic twins[133].

The serotonin transporter (*SERT*) gene encoding the SERT protein is located on chromosome 17q11.1-q12. A functional polymorphism is the insertion or a deletion of 44 base pairs in the *SERT*-gene-linked polymorphic region[134]. An association was reported between a functional polymorphism in the *SERT* gene and diarrhoea-predominant IBS[135,136]. Individuals with a long allele genotype of the *SERT* gene have been shown to be vulnerable to developing IBS with constipation[137]. Other studies, however, did not show such association between *SERT*-gene polymorphism and IBS[136]. A polymorphism in the CCK1 receptor *CCKAR* gene (779T>C) has also been found to be associated with IBS[138,139].

Environment and social learning

Parental modelling and the reinforcement of illness behaviour can contribute to the causes of IBS[140-144]. Having a mother with IBS has been shown to account for as much variance as having an identical set of genes as a co-twin who has IBS. This suggests that the contribution of social learning to IBS is at least as great as the contribution of heredity[144].

Dietary and intestinal flora

Patients with IBS believe that their diet has a significant influence on their symptoms and they are interested in finding out which foods they should avoid[145-148]. About 60% of IBS patients report a worsening of symptoms following food ingestion: 28% within 15 min after eating and 93% within 3 h[148]. Many IBS patients report specific foods as triggers, most commonly implicating milk and dairy products, wheat products, onion, peas and beans, hot spices, cabbage, certain meats, smoked products, fried food and caffeine as the offending foods[149]. However, dietary composition among IBS patients in the community does not differ from community controls[150-153]. In a recent study, IBS patients were reported to have made a conscious choice to avoid certain food items, some of which belong to fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs). However, they reported a higher consumption of other food items that are rich in FODMAPs. Patients also reportedly avoided other food sources that are important for health, which result in a low intake of calcium, phosphorus and vitamin B2[153].

There is no documented evidence showing that a food allergy or intolerance plays a role in IBS symptoms[1]. The reaction of IBS patients to certain food items has been attributed to a number of short-chain carbohydrates that are poorly absorbed so that a significant portion of the ingested carbohydrates enters the distal small bowel and colon. Once there they increase the osmotic pressure and provide a substrate for bacterial fermentation with the production of gas, distension of the large intestine and abdominal discomfort or pain. These carbohydrates are FODMAPs and include fructose, lactose, fructans, galactans and sugar alcohols, such as sorbitol, maltitol, mannitol, xylitol and ismalt. Fructose and lactose are present in apples, pears, watermelon, honey, fruit juices, dried fruits, milk and dairy products. Polyols are used in low calorie food products. Galactans and fructans are present in common dietary constituents, such as wheat, rye, garlic, onions, legumes, cabbage, artichokes, leeks, asparagus, lentils, inulin, soy, Brussels sprouts and broccoli[78,147].

A deficiency in dietary fibre was widely believed to be the primary cause of IBS[154]. Although increasing the amount of dietary fibre continues to be a standard recommendation for patients with IBS, clinical practice has shown that increased fibre intake in these patients increases abdominal pain, bloating and distension. IBS patients assigned to the fibre treatment showed persistent symptoms or no improvement in symptoms after treatment compared to patients taking the placebo or a low-fibre diet. Other studies have shown that whilst a water-insoluble fibre intake did not improve IBS symptoms, soluble-fibre intake was effective in improving overall IBS symptoms[155,156]. It is noteworthy that the role of FODMAPs and fibre on IBS symptoms is associated with intestinal flora. The presence of bacteria that break down FODMAPs and fibre and produce gas, such as *Clostridia spp.*, can cause distension of the large intestine with abdominal discomfort or pain.

Most bacteria in the GI tract exist in the colon. The colon of each individual contains between 300 and 500 different species of bacteria[1], and each person has his own unique intestinal flora. The intestinal flora is affected by several factors, such as diet, climate changes, stress, illness, aging and antibiotic treatment[1]. The intestinal flora in IBS patients has been found to differ considerably from that of healthy controls, as IBS patients have fewer *Lactobacillus* and *Bifdobacterium spp.* than healthy subjects[157]. These bacteria bind to epithelial cells and inhibit pathogen binding as well as enhancing barrier functioning[158]. Furthermore, these bacterial species do not produce gas upon fermenting carbohydrates, which is an effect that would be amplified as they also inhibit the *Clostridia spp.*[158]. Probiotics alter colonic fermentation and stabilise the colonic microbiota, and several studies on probiotics have shown improvements in flatulence and abdominal distension, with a reduction in the composite IBS symptom score[158-160].

Low-grade inflammation

In a subset of IBS patients GI symptoms appear following gastroenteritis, with about 25% of patients showing IBS-D symptoms 6 mo post-infection and approximately 10% developing persistent symptoms[161-164]. Post-infectious (PI)-IBS has been reported after viral, bacterial, protozoa and nematode infections[1], with the incidence of PI-IBS varying between 7% and 31%, although the largest studies suggest this number is about 10%[161-164]. One study showed that 6% to 17% of sporadic (un-

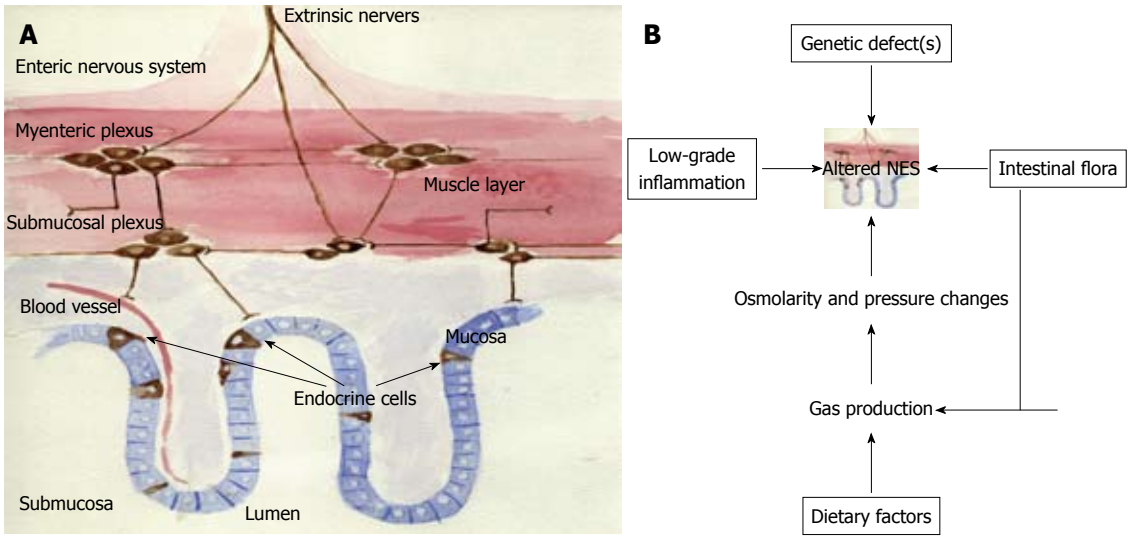


Figure 4 Schematic drawing to illustrate the neuroendocrine system of the gut and the possible pathogenesis of irritable bowel syndrome. A: Schematic drawing of neuroendocrine system; B: Possible pathogenesis of irritable bowel syndrome. Reproduced from reference [1] with permission from Nova Science Publisher, Inc. NES: Neuroendocrine system.

selected) IBS patients believed that their symptoms began with an infection[1]. Following infection, the initial inflammatory response shows an increase in CD3 lymphocytes, CD8 intraepithelial lymphocytes and calprotectin-positive macrophages[161]. These changes rapidly decrease in most subjects but a small number with persistent symptoms fail to show this decline[165]. Furthermore, the number of serotonin cells was shown to increase in subjects with persistent symptoms[165]. There are several pieces of evidence showing that inflammation and immune cells affect the NES of the gut, which controls and regulates GI motility and sensitivity[166]. Thus, serotonin secretion by enterochromaffin (EC) cells can be enhanced or attenuated by the secretory products of immune cells such as CD4+T[167]. Furthermore, serotonin modulates the immune response[167]. The EC cells are in contact with or very close to CD3+ and CD20+ lymphocytes, and several serotonergic receptors have been characterised in lymphocytes, monocytes, macrophages and dendritic cells[168]. Moreover, immune cells in the small and large intestine show receptors for substance P and vasoactive intestinal polypeptide[169].

IBS occurs in 32%-46% of patients with ulcerative colitis (UC) and in 42%-60% of Crohn's disease patients who are in remission[170-174]. Faecal calprotectin has been found to be significantly elevated in UC and Crohn's disease patients with criteria for IBS, compared to those without IBS-type symptoms, indicating the presence of occult inflammation[174].

Abnormalities in the NEC of the gut in IBS

The NES of the gut consists of two parts: endocrine cells scattered among the epithelial cells of the mucosa facing the gut lumen, and peptidergic, serotonergic and nitric oxide-containing nerves of the enteric nervous system (ENS) in the gut wall (Figure 4A)[1]. This system regulates several functions of the GI tract, such as motility, secretion, absorption, microcirculation in the gut, local immune defence and cell proliferation[1]. This regulatory system includes a large number of neuroendocrine peptides/amines, which exert their effects *via* a number of actions: an endocrine mode of action, by circulating in the blood to reach distant targets, an autocrine/paracrine mode, which is a local action, and *via* synaptic signalling or *via* neuroendocrine means, which involve the release from synapses into the circulating blood. The different parts of this system interact and integrate with each other and with afferent and efferent nerve fibres of the central nervous system, in particular the autonomic nervous system. There are at least 14 different populations of endocrine or paracrine cells in the GI tract[1]. The ENS comprises a large variety of neurotransmitters and associated receptors. Almost every known neurotransmitter can be found in the ENS, and most of the receptors associated with these neurotransmitters are also expressed there[1].

In the stomach of patients with IBS, the density of ghrelin-immunoreactive cells in the oxyntic mucosa was found to be significantly lower in IBS-constipation patients and significantly higher in IBS-diarrhoea patients compared to healthy controls[175]. However, the levels of total or active ghrelin in plasma and stomach tissue extracts from IBS patients did not differ from those of healthy subjects[175,176]. Ghrelin is a 28-amino acid peptide hormone that was originally isolated from the stomach[177]. Ghrelin mostly originates from endocrine cells in the oxyntic mucosa of the stomach, but small amounts are expressed in the small intestine, large intestine and in the arcuated nucleus of the hypothalamus[177]. Ghrelin has several functions, including a role in regulating growth hormone (GH) release from the pituitary, where it acts synergistically with the GH-releasing hormone[178,179]. Ghrelin also increases appetite and feeding and plays a major role in energy metabolism[178-181]. Furthermore, this hormone has been found to accelerate gastric and

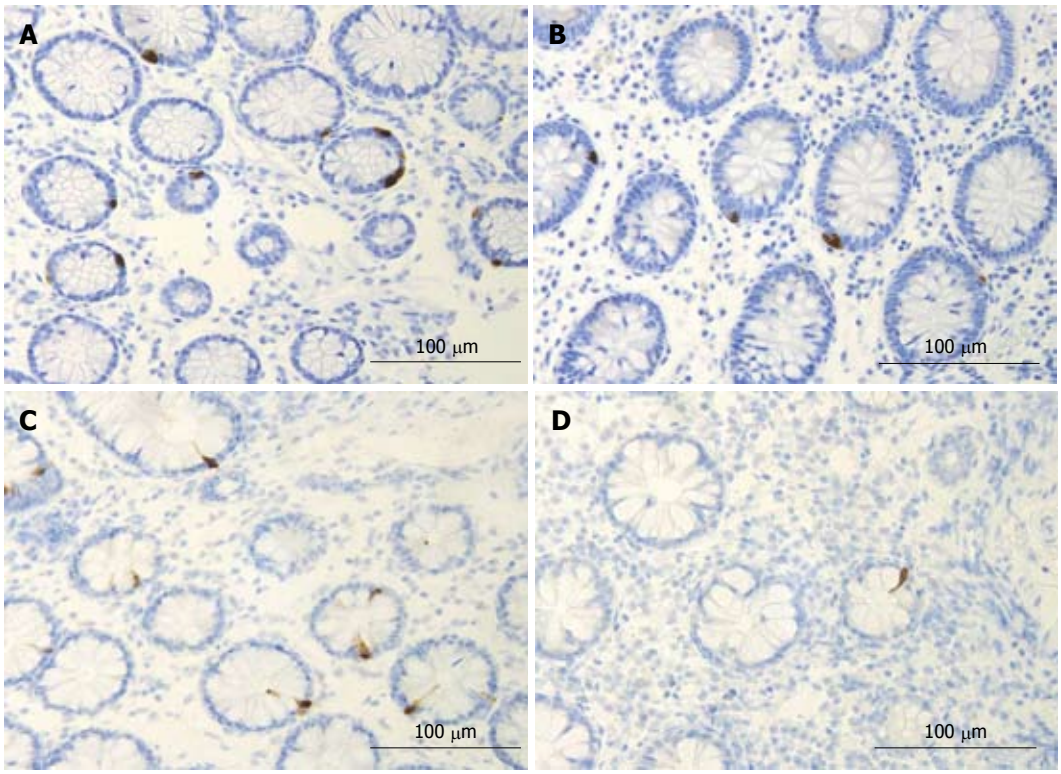


Figure 5 Serotonin cells and polypeptide YY immunoreactive cells in the colon. A: A healthy control in serotonin cells; B: A patient with irritable bowel syndrome in serotonin cells; C: A healthy subject in polypeptide YY (PYY) immunoreactive cells; D: An irritable bowel syndrome patient in PYY immunoreactive cells. Reproduced from reference 1 with permission from Nova Science Publisher, Inc.

small and large intestinal motility[181-192], as well as having anti-inflammatory actions and protecting the gut against a wide range of insults. The density of neuropeptide-expressing cells is altered in the small intestine of IBS patients. Thus, the density of cells expressing gastric inhibitory polypeptide and somatostatin is decreased in patients with both diarrhoea- and constipation-predominant IBS subtypes[193]. The densities of secretin and cholecystokinin (CCK)-expressing cells are decreased in the diarrhoea-predominant subtype, but not in the constipation-predominant subtype. Serotonin cell density has also been found to be unchanged in the duodenum of IBS patients, regardless of the subtype[193], which is interesting as serotonin cells were previously reported to be affected in the small intestine of IBS patients[194-196]. These peptides all play important roles in secretion and gastric motility. In the large intestine, serotonin and polypeptide YY (PYY) cell densities have been found to be low in both IBS-constipation and IBS-diarrhoea patients (Figure 5)[197]. Furthermore, the mucosal 5-HT concentration has also been reported to be low in IBS patients[197], which is in line with current observations. In PI-IBS, the number of CCK and serotonin cells has been reported to be increased in the small intestine[198], and serotonin and PYY cell numbers were found to be increased in the large intestine[199-202].

HYPOTHESIS

As described above, abnormalities in the neuroendocrine peptides/amines of the gut have been reported. These abnormalities could cause disturbances in digestion, GI motility and visceral hypersensitivity. These abnormalities appear to contribute to symptom development and could play a central role in the pathogenesis of IBS. Genetic differences have been found between IBS patients and healthy subjects in genes controlling the serotonin signalling system and CCK. Moreover, differences in the diet, intestinal flora and inflammation affect the NES of the gut. The release of different gut hormones depends on the composition and quantity of ingested food, as the food content of FODMAPs and fibre, intestinal flora and the subsequent fermentation can increase intestinal osmotic pressure. This change in intestinal pressure can stimulate hormonal release, such as the release of serotonin. Likewise, inflammation and the release of secretory products from immune cells effects hormonal release and the proliferation of gut endocrine cells.

Therefore, it is feasible to hypothesise that the cause of IBS is an altered NES (Figure 4B). An altered NES would cause abnormal GI motility, secretion and sensation, all of which are characteristic of IBS[203-216]. The alteration in NES could be a result of one or more of the following: genetic factors, dietary intake, intestinal flora or low-grade inflammation.

CONCLUSION

The diagnosis of IBS is based on symptom assessment and the Rome Ⅲ criteria. Whereas the latter has been

widely used in scientific studies and in GI congresses in the past 10 years, it is not, however, used by most clinicians consulted by IBS patients[217-220]. This is not because these clinicians are unaware of the Rome Ⅲ criteria, but because of the reality in the clinic. IBS patients that seek advice from a doctor are worried and want to be investigated, and are rarely satisfied until this is done, so they will repeatedly seek healthcare until they are investigated. I believe, therefore, that the Rome Ⅲ criteria should be combined with a physical examination, blood tests, gastroscopy, duodenal biopsies and colonoscopy with segmental biopsies. These examinations and tests, in addition to the Rome Ⅲ criteria, would reassure the patient and exclude CD, IBD, MC and cancer. Furthermore, performing these examinations and tests would remove the pressure applied by some patients to perform these examinations repeatedly, as the need for further investigations can always be argued against if there are no new symptoms. Duodenal chromogranin A cell density also appears to be a promising biomarker for the diagnosis of IBS.

The pathogenesis of IBS appears to be multifactorial. There is evidence to suggest that the following factors play a central role in the pathogenesis of IBS: heritability and genetics, dietary and intestinal microbiota, low-grade inflammation and disturbances in the NEC of the gut. Several authors have tried to connect these factors in a logical cause-effect pattern, but it is my belief that the proposed hypothesis presented in this review is the most logical.

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GUIDELINES FOR CLINICAL PRACTICE

Gastric carcinogenesis

Ismail Gomceli, Baris Demiriz, Mesut Tez

Ismail Gomceli, Department of Gastroenterologic Surgery, Antalya Education and Research Hospital, 07070 Antalya, Turkey
Baris Demiriz, Mesut Tez, Department of General Surgery, Ankara Numune Education and Research Hospital, 06100 Ankara, Turkey
Author contributions: Gomceli I, Demiriz B and Tez M reviewed the literature and wrote the paper.
Correspondence to: Mesut Tez, MD, Department of General Surgery, Ankara Numune Education and Research Hospital, 5. cadde 10/3 Bahcelievler, 06100 Ankara, Turkey. mtez@hacettepe.edu.tr
Telephone: +90-532-4160669 Fax: +90-312-3061443
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Abstract

Gastric cancer is the second most common cancer worldwide and the second most common cause of cancer-related deaths. Despite complete resection of gastric cancer and lymph node dissection, as well as improvements in chemotherapy and radiotherapy, there are still 700 000 gastric cancer-related deaths per year worldwide and more than 80% of patients with advanced gastric cancer die of the disease or recurrent disease within 1 year after diagnosis. None of the treatment modalities we have been applying today can influence the overall survival rates: at present, the overall 5-year relative survival rate for gastric cancer is about 28%. Cellular metaplasia due to chronic inflammation, injury and repair are the most documented processes for neoplasia. It appears that chronic inflammation stimulates tumor development and plays a critical role in initiating, sustaining and advancing tumor growth. It is also evident that not all inflammation is tumorigenic. Additional mutations can be acquired, and this leads to the cancer cell gaining a further growth advantage and acquiring a more malignant phenotype. Intestinalization of gastric units, which is called "intestinal metaplasia"; phenotypic antralization of fundic units, which is called "spasmolytic polypeptide-expressing metaplasia"; and the development directly from the stem/progenitor cell zone are three pathways that have been described for gastric carcinogenesis. Also, an important factor for the development of gastrointestinal cancers is peritumoral stroma. However, the initiating cellular event in gastric metaplasia is still controversial. Understanding gastric carcinogenesis and its precursor lesions has been under intense investigation, and our paper attempts to highlight recent progress in this field of cancer research.

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Key words: Gastric Cancer; Cancer Stem Cell; Carcinogenesis; Oncogenesis; Tumorigenesis

Peer reviewers: Dr. Wael El-Rifai, Department of Surgery, Vanderbilt University Medical Center, 1255 MRB-IV Light Hall, 2215 Garland Ave, Nashville, TE 37232, United States; Shogo Kikuchi, Professor, Department of Public Health, Aichi Medical University School of Medicine, 21 Karimata, Yazako, Nagakute-cho, Aichi 480-1195, Japan

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GASTRIC CARCINOMA

Cancer is a major public health problem and at the beginning of the 19th century, gastric cancer was the second most common cancer worldwide[1]. Every year there are 900 000 new cases and 700 000 gastric cancer-related deaths in the world[2]. Although chemotherapy improves life expectancy, and despite seemingly complete resection of gastric cancer (R0) *via* gastrectomy, more than 80% of patients with advanced gastric cancer die of the disease or recurrent disease within 1 year after diagnosis. This situation suggests that standard treatment protocols are ineffective in a considerable number of cases[3]. Thus, the understanding of the mechanism underlying the progression of gastric carcinoma is essential for the management of this disease.

RISK FACTORS

A number of risk factors are known for gastric cancer (Table 1), but study results regarding some factors, especially salt intake, vitamin C, alcohol, occupational exposure to nitrosamines and inorganic dusts, have been inconsistent[4-9].

HISTOLOGY AND PATHOLOGY

The majority of gastric cancer patients have adenocarcinoma (90%); the remaining 10% have lymphoma or gastrointestinal stromal tumor. There are two general types of gastric adenocarcinoma: the intestinal type (50%) and the diffuse type (33%) according to the Lauren classification system[9]. The remaining 17% are mixed or unclassified type[10]. The intestinal type is more common and is more often located in the distal part of the stomach. In contrast, the diffuse type has a poorer prognosis; generally occurs in younger patients; and can occur anywhere in the stomach, but especially in the cardia. The intestinal type is frequently accompanied by liver metastasis, whereas because the diffuse type has an increased propensity for intra- and trans-mural spread, it has been associated with peritoneal dissemination and poorer prognosis[1]. The diffuse type of gastric cancer shows more poorly differentiated cells than the intestinal type[11]. Intestinal-type adenocarcinoma is preceded by metaplastic changes, whereas diffuse-type adenocarcinoma is thought to arise in normal gastric mucosa.

Gastric adenocarcinoma can also be divided into two groups, known as "differentiated" and "undifferentiated", using the Nakamura classification system[12]. Intestinal-type adenocarcinoma is considered to be essentially equivalent to differentiated adenocarcinoma, as is diffuse-type equivalent to the undifferentiated adenocarcinoma. However, some cases of intestinal-type adenocarcinoma also arise from the gastric mucosa without intestinal metaplasia (IM). So based on the type of IM, some authors suggest that gastric cancer phenotypes can be classified into four groups depending on the marker combinations as: complete intestinal type, incomplete intestinal type, gastric type and unclassified type. Gastric-type differentiated adenocarcinomas can be distinguished from other types of differentiated adenocarcinomas on the basis of their increased malignant potential in the incipient phase of invasion and metastasis[13].

The mucous epithelium of the stomach represents a major barrier to the various noxious agents by means of intercellular tight junctions. This epithelium and its components are also vital for complex communications and physiological functions[14]. Histologically, the human gastric mucosa is divided into three regions: cardia, fundus-corpus and antrum-pylorus. Also, a transitional zone separates the stereotypic corpus and antral/pyloric epithelia and has features of each. The epithelium of these regions is composed of millions of glands that are surrounded by supporting stromal cells which are derived from mesenchyme. In the corpus, glands are long and composed of several epithelial cell types, including surface mucous foveolar cells (pit cells), acid-making oxyntic (parietal) cells, mucous neck cells (intermediate progenitor for chief cells), zymogenic (chief) cells, and hormone-secreting endocrine cells. In the antrum, the shorter glands are composed mainly of mucus-secreting cells and endocrine cells that secrete hormones such as gastrin and somatostatin. The stomach mesenchymal compartment surrounding the glands is less studied and little understood[15-18].

The human stomach mucosal tubular glands are further subdivided into foveolus, isthmus, neck and base regions. The gastric glands open into the bottom of the pits, on an average with 4 to 5 glands per pit. Fundic glands are quite straight, whereas antral glands are branched and coiled in their basal ends. Fundic and antral units (combination of a pit and a gland) differ very much in their cell characteristics and turnover rates (the human antral mucosa is known to have a much higher turnover rate). The gastric glands which contain 'surface mucous' cells and "mucous neck" cells (in the foveola), pepsinogen-secreting zymogenic (chief) cells (at the base of the glands), acid-secreting oxyntic (parietal) cells (at the base of the glands), and endocrine cells including the histamine-producing enterochromaffin-like (ECL) cells are located in the fundus; the zymogenic (chief) cells, oxyntic (parietal) cells and ECL cells are also found in the corpus of the stomach. The antral unit contains surface mucous foveolar cells, antral gland cells, endocrine cells (mainly gastrin-producing G-cells, but also EC and somatostatin-producing D cells), and occasional oxyntic cells. In the pylorus, the gastric glands contain many more mucinous cells, no zymogenic cells and few oxyntic cells (Figure 1)[19-23].

In addition, it should be noted that the subepithelial mesenchymal cells and their secreted basement membrane factors compose the lamina propria. This constitutes a structural support while regulating epithelial cell function and epithelial cell networks[24,25].

MOLECULAR TARGETS AND SIGNALING PATHWAYS

Some of the earliest observations in cancer biology as well as recent advances in molecular analyses contribute to our knowledge about the multistep process of gastric carcinogenesis[26-28].

The gastrointestinal tract has rapid epithelial turnover and exposure to injury by infections and dietary toxins. These conditions create very high cancer prevalence. Intestinalization of gastric units, which is called "IM"; phenotypic antralization of fundic units, which is called "spasmolytic polypeptide-expressing metaplasia (SPEM)"; and the development directly from the stem/progenitor cell zone, are three pathways that have been described for gastric carcinogenesis[29-31].

Neoplasia can follow cellular metaplasia due to chronic inflammation, injury and repair[32]. This is the most documented process for gastric cancer[33-35]. An accept-

Table 1 Risk factors for gastric cancer

Genetic factors	Environmental factors	Other factors
Sex	*Helicobacter pylori*	Gastric adenomas
Familial adenomatous polyposis	Epstein-Barr virus	Barrett's esophagus
Hereditary nonpolyposis colorectal cancer (Lynch Ⅱ)	Nitrites	Hamartomas
Genetic diffuse gastric cancer (E-cadherin - CDH1 mutation)	Excess alcohol ingestion	Ménétrier's disease
Genetic polymorphisms for pro- and anti-inflammatory cytokines	High intake of salted, pickled, or smoked foods	Chronic atrophic gastritis
Polymorphisms for cell receptors of innate immune response	Low intake of fiber, fruits and vegetables	Gastric metaplasia
Peutz-Jeghers syndrome	Antioxidant consumption (especially ascorbic acid, carotenoids, folates and tocopherols)	Pernicious anemia
	Tobacco smoking (adenocarcinoma of cardia)	Benign gastric ulcers
		Fundic gland polyps
		Hyperplastic polyps
		Gastric biopsy revealing high-grade dysplasia
		History of subtotal gastrectomy (> 20 yr)

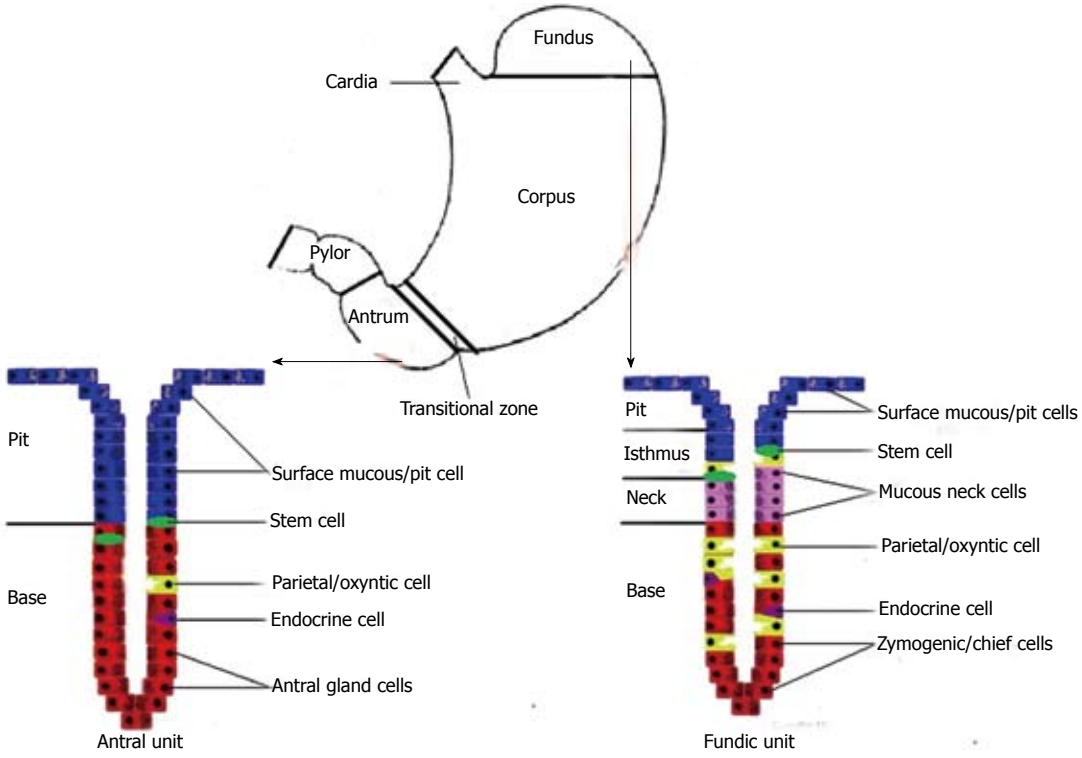


Figure 1 Schematic explanation of fundic and antral units.

able concept is that there are two corner-stones with regard to this process. Firstly, the initial observation of Rudolf Virchow in 1863 about leucocytes in neoplastic tissues and the connection between inflammation and cancer[36]; secondly, about 15 years ago, researchers' evidence about the relationship between stomach cancer and infection by *Helicobacter pylori* (*H. pylori*) (isolated by Drs. Marshall and Warren in 1984[37]. Also, we must note that Epstein-Barr virus has been detected in stomach tissues in approximately 10% of gastric carcinoma cases[38].

Beginning with some of the earliest observations in cancer biology, it appears that chronic inflammation stimulates tumor development and plays a critical role in initiating, sustaining and advancing tumor growth[39,40]. Direct effect of the viral pathogens on neoplastic transformation of epithelial cells has been shown; however, it is also evident that not all inflammation is tumorigenic[41]. It can be suggested that either the tumor alters the immune response by reactive oxygen species and cytokines or chronic inflammation plays a primary role in transforming tissue cells (especially mentioned in "stem cell theory") into tumor cells. In the acute phase of inflammation, the release of endogenous reactive oxygen and nitrogen species (O_2^-, H_2O_2, NO, OH, $ONOO^-$, HOCl) from such innate immune cells as macrophages and leukocytes plays an important role in the elimination of

pathogens[42]. However, when present chronically, this can induce DNA damage in proliferating cells. In addition, it is also possible for other bacteria to colonize the stomach and additionally trigger carcinogenesis by gastric atrophy (result of chronic inflammation) which represents a loss of gastric glands and associated lower acidity of gastric juice[43,44]. Hypoacidity associated with *H. pylori* infection induces gastric mucosal atrophy to advance multistage carcinogenesis in the stomach. Interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are elevated in gastric mucosa with *H. pylori* infection. Gastrin is upregulated and acid secretion from parietal cells is inhibited mainly due to pro-inflammatory cytokines IL-1β and TNF-α[45,46]. TNF-α and IL-1β are essential in the initiation of chronic inflammation. Recent works have shown that IL-1β overexpression, in the absence of *Helicobacter* infection, is sufficient to cause gastric cancer and it is one of the essential proinflammatory cytokines modulated during *H. pylori* infection that directs the mucosa toward atrophy, metaplasia, and neoplastic transformation[47-49]. Another important point that should be added is that *H. pylori* has been consistently associated with higher risk of gastric noncardia cancer. The inverse association of *H. pylori* with gastric cardia cancer or esophageal adenocarcinoma has been shown in several studies, especially in Western populations[50]. Furthermore, mast cells in particular play an important role in attracting inflammatory cells by releasing inflammatory mediators. Monocytes differentiate into macrophages, and become activated in response to local chemokine and cytokine interactions[51]. Also, the correlation between tumor-associated macrophage abundance and poor prognosis has been shown[52]. Furthermore, macrophage-deficient mice display reduced progression of tumors to a more malignant phenotype[53]. Recently, direct evidence has also linked IL-6 to inflammation-mediated tumor initiation and proliferation in colon cancer[54]. IL-6 can inhibit dendritic cell maturation and, together with the NF-κB-activating cytokines IL-1 and TNF, can promote tumor progression. Cytokines also affect cell death and cell cycle pathways[55,56]. TNF-α is produced mainly by macrophages. It is also produced by tumor cells. TNF-α is associated with tissue destruction and plays a role in destroying tumor blood supply. However, if it is produced chronically, it can act as a tumor promoter by contributing to tissue remodeling and stromal development[57,58]. Nuclear factor (NF)-κB and STAT3 pathways have emerged as key regulators of the release of these pro-inflammatory cytokines, and important mediators of both tumor proliferation and persistence of chronic inflammation. The activation of these pathways results in further cytokine release[57,59,60].

Activation of the innate immune system is followed by the adaptive immune response. Th1 response and its accompanying mediators (IFN-γ) are not only necessary for *Helicobacter*-induced inflammation but also for the development of atrophy or metaplasia and SPEM; however a Th2 response and its mediators (i.e., IL-4) appear to be protective. The presence of a Th1, rather than a Th2, immune response is also associated with better survival in gastric cancer patients[36].

Although the subsequent pathways are different, chronic inflammation is the first step in both the intestinal and the diffuse type of gastric cancer. While the intestinal type has a sequence of multifocal atrophic gastritis, IM and dysplasia, which advances to carcinoma, the diffuse type tends to be primarily genetic in origin[61,62]. The progress from IM to gastric cancer has a wide range of molecular alterations affecting transcription factors, such as CDX1 and CDX2, telomerases, microsatellite instability, mutations of p53 protein, overexpression of COX-2, cyclin D2, and decreased expression of p27[63]. The next step is gastric dysplasia. During the progression of normal tissue through the metaplasia-dysplasia sequence, there are mutations in genes including *p53*, also loss of heterozygosity of the adenomatous polyposis coli gene, overexpression of the antiapoptotic gene *bcl-2* and a mixture of polyploidy and aneuploidy[63].

Inflammation also plays an important role in the ability of tumor cells to invade and metastasize. The ability of epithelial tumor cells which metastasize to express specific chemokine receptors has been shown[64]. Paracrine secretion of pro-inflammatory cytokines (i.e., IL-1β, IL-6, TNF-α) and certain autocrine cytokine production support this process[65]. During the later stages, additional mutations can be acquired, and this leads to the cancer cell gaining a further growth advantage and acquiring a more malignant phenotype[66,67].

THERAPEUTICS AND OUTCOME

In recent studies investigators have found out that *K-ras* activation resulted in an inflammatory response and enhanced the expression of COX-2 in the glandular stomach. COX2 is upregulated in the gastric epithelium and in the infiltrating inflammatory cells in the stomach during gastritis[68-70]. Furthermore, it has been shown that sulindac, a nonsteroidal anti-inflammatory drug, suppresses the progression of gastric cancer in mice[71]. Hence, a *K-ras* activation-induced inflammatory response may facilitate the formation of IM and promote the progression of gastric cancer.

SPEM is associated more commonly with gastric cancer than IM[72,73]. It can be defined as a corpus lesion. Nevertheless, IM and SPEM often occur together[74,75]. Increase in mucus and loss of mature parietal and chief cells in humans correlates with SPEM (Figure 2)[73]. SPEM is characterized by expression of TFF2 (spasmolytic polypeptide) which is normally a product of mucous neck cells and antral gland cells[72]. SPEM also arises from a second proliferative zone at the bases of metaplastic fundic units, either by transdifferentiation of chief cells or activation of an unknown basal crypt progenitor[76,77]. However, it is not clear whether these cells are related to the gastric progenitor cells[78].

It must be noted that an important factor for the development of gastrointestinal cancers is peritumoral stro-

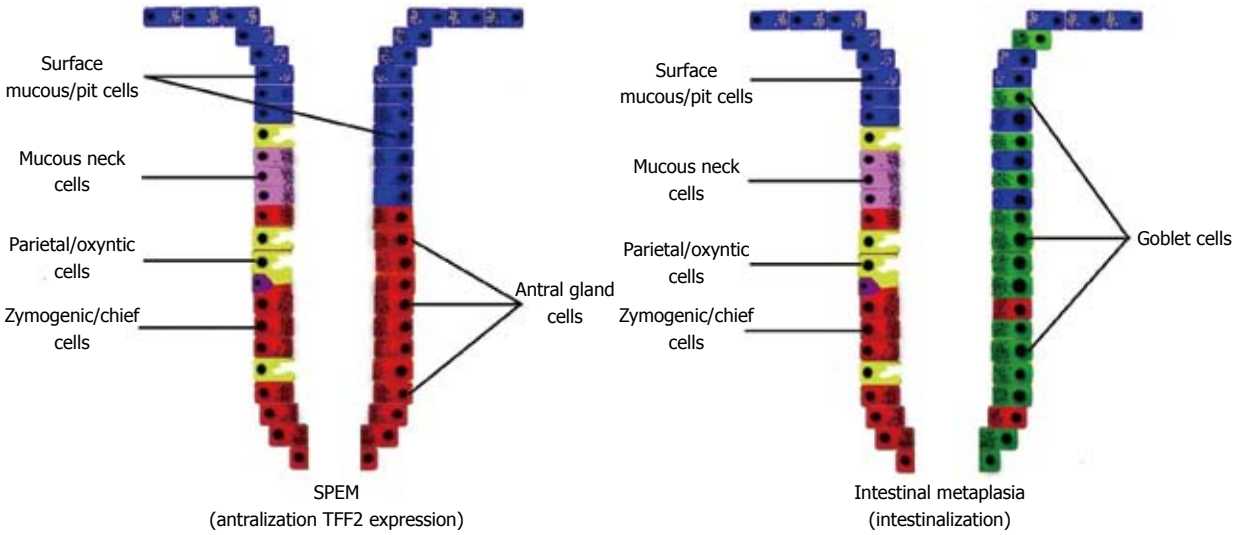


Figure 2 Schematic explanation of spasmolytic polypeptide-expressing metaplasia and intestinal metaplasia. SPEM: Spasmolytic polypeptide-expressing metaplasia; TFF-2: Trefoil family factor 2.

ma. Activated fibroblasts within the stroma can help to create an environment containing vessels and infiltrating inflammatory cells and it is the interaction between these different cell types which is permissive of tumor growth, angiogenesis, and invasion[79-81].

The question that must be answered is: what is the initiating cellular event in gastric metaplasia? The interpretation that the metaplasia is an intermediate step in the development of gastric cancer may be facile, because different types of IM have different degrees of association with malignancy, and early stage gastric cancers can arise in nonintestinalized epithelium[82-84]. Investigators have reported that solid cancers might originate from differentiated cells and they have reported the possible existence of cancer stem cells (CSCs) or tumor initiating cells in solid malignant tumors[85,86]. However, based on the assessment of the differentiation status of tumor cells, they appear to deviate little from their normal progenitors and to show similar differentiation programs. Studies on tissues undergoing continuous cell renewal suggest that cancer cells may originate from a stem cell compartment[87]. The origin of human gastric CSCs has yet to be elucidated, but data obtained from a mouse model of *Helicobacter*-induced gastric cancer have implicated bone marrow-derived cells as a potential candidate. Further studies focusing on the identification and characterization of CSCs in gastric cancer may lead to novel diagnostic and therapeutic tools, dramatically improving the prognosis of gastric cancer patients.

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REVIEW

KRAS mutation testing in metastatic colorectal cancer

Cong Tan, Xiang Du

Cong Tan, Xiang Du, Department of Oncology, Shanghai Medical School, Fudan University, Shanghai 200032, China
Cong Tan, Xiang Du, Department of Pathology, Shanghai Cancer Center, Fudan University, Shanghai 200032, China
Author contributions: Tan C wrote this manuscript; and Du X significantly designed and revised the manuscript, and approved the final version.
Supported by Science and Technology Commission of Shanghai Municipality, No. 10DJ1400501
Correspondence to: Xiang Du, MD, PhD, Department of Pathology, Shanghai Cancer Center, Fudan University, 270 Dong An Road, Shanghai 200032, China. dx2008cn@yahoo.com.cn
Telephone: +86-21-64175590 Fax: +86-21-64170067
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Abstract

The *KRAS* oncogene is mutated in approximately 35%-45% of colorectal cancers, and *KRAS* mutational status testing has been highlighted in recent years. The most frequent mutations in this gene, point substitutions in codons 12 and 13, were validated as negative predictors of response to anti-epidermal growth factor receptor antibodies. Therefore, determining the *KRAS* mutational status of tumor samples has become an essential tool for managing patients with colorectal cancers. Currently, a variety of detection methods have been established to analyze the mutation status in the key regions of the *KRAS* gene; however, several challenges remain related to standardized and uniform testing, including the selection of tumor samples, tumor sample processing and optimal testing methods. Moreover, new testing strategies, in combination with the mutation analysis of *BRAF*, *PIK3CA* and loss of *PTEN* proposed by many researchers and pathologists, should be promoted. In addition, we recommend that microsatellite instability, a prognostic factor, be added to the abovementioned concomitant analysis. This review provides an overview of KRAS biology and the recent advances in *KRAS* mutation testing. This review also addresses other aspects of status testing for determining the appropriate treatment and offers insight into the potential drawbacks of mutational testing.

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Key words: *KRAS*; Epidermal growth factor receptor; Metastatic colorectal cancer; Testing status; Biomarker

Peer reviewers: Ulrike Susanne Stein, PhD, Assistant Professor, Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Straße 10, 13125 Berlin, Germany; Dr. John B Schofield, MB, BS, MRCP, FRCP, Department of Cellular Pathology, Preston Hall, Maidstone, Kent ME20 7NH, United Kingdom

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide. In the United States, approximately 102 900 cases of colon cancer and 39 670 cases of rectal cancer were diagnosed in 2010, and approximately 51 370 patients died of CRC in the same year, accounting for about 9% of all cancer deaths[1]. With the emergence of two anti-epidermal growth factor receptor (EGFR)-targeted antibodies, cetuximab (Erbitux) and panitumumab (Vectibix), the treatment of metastatic CRC has entered into the era of personalized treatment. Of the two antibodies, one is a human-mouse chimeric IgG1 monoclonal that was approved by the United States Food and Drug Administration (FDA) in 2004 as a second-line treatment of CRC; the other is a human IgG2 k monoclonal antibody that was approved by the FDA as a third-line drug in 2007. However, EGFR, the target of these drugs, which is overexpressed in approximately 80% of colorectal carcinomas, failed to predict a therapeutic response when used clinically[2,3]. Therefore, downstream signaling effectors were sought to help predict the efficacy of anti-EGFR treatment. The *KRAS* gene, which has

been extensively studied for more than three decades, has been demonstrated to be a strong negative predictive biomarker to indicate whether a CRC patient will respond to anti-EGFR treatment. As the target treatment may also be toxic and expensive, *KRAS* mutation status detection has become a crucial diagnostic factor for treating metastatic CRC patients.

KRAS GENE AND ITS ROLE IN EGFR SIGNALING

The *RAS* gene was initially identified as a viral gene homologous to the transforming gene from the Kirsten rat sarcoma virus[4,5]. Mutations in *RAS* are found in approximately 30% of all human cancers, making it one of the most commonly mutated genes in cancer[6]. The KRAS protein, also called p21, is a member of the *Ras* superfamily of proteins, is located on human chromosome 12 and encoded by 189 amino acids, and contains four coding exons and a 5' non-coding exon[7]. KRAS is a membrane-anchored guanosine triphosphate/guanosine diphosphate (GTP/GDP)-binding protein and is widely expressed in most human cells. As a small GTPase (GTP cleaving enzyme), KRAS is involved in intracellular signal transduction and mainly responsible for EGFR-signaling activation. The exchange of the active GTP-bound state and the inactive GDP-bound state is tightly controlled by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors[8]. Under normal physiological conditions, upstream signals activate wild-type *KRAS* by promoting the exchange of bound GDP for GTP. This process is transient because of GAP-mediated GTP hydrolysis. However, this process becomes altered when the *KRAS* gene is mutated.

Mutant *KRAS* is found in about 35%-45% of CRCs[9-15], and codon 12 and 13 are two hotspots, which account for about 95% of all mutation types, with approximately 80% occurring in codon 12 and 15% in codon 13. Other mutations in codons 61, 146 and 154 occur less frequently in CRC, accounting for 5% of all mutation type[16]. Referring to the Catalogue of Somatic Mutations in Cancer Database, more than 5000 mutations have been found in the *KRAS* gene in CRC samples.

KRAS mutations are almost single nucleotide point mutations as reported, and the most common patterns are G12D, G12A, G12R, G12C, G12S, G12V and G13D. In the codon 12 mutation, p.G12D, pG12V is the most frequent, and in codon 13, the substitution of glycine for aspartate (p.G13D) is the most frequent[17].

These mutations impair the intrinsic GTPase activity of KRAS and prevent GAPs from promoting GTP hydrolysis by KRAS, therefore causing KRAS proteins to accumulate in the GTP-bound, active form. In this manner, mutant *KRAS* results in a constitutively active GTP-bound state and the activation of downstream proproliferative signaling pathways[18,19]. Therefore, *KRAS* mutations play a critical role in human tumorigenesis and are the most prevalent in pancreatic, thyroid, colorectal and lung cancers.

SIGNIFICANCE OF *KRAS* MUTATION TESTING

KRAS as a prognostic factor

It has been suggested that prognostic and predictive factors should be clarified; the former (including traditional clinical markers like lymph node involvement, the histological grade of the tumor, and molecular biomarkers, *etc*.) often refers to the outcome of the natural history of the tumor, while the latter predicts the response to the therapies. Until recently, the prognostic value of *KRAS* mutation was in dispute. Two canonical trials have demonstrated that the *KRAS* mutation may be prognostic of treatment outcomes for patients with CRC. The Kisten Ras in Colorectal Cancer Collaborative Group Study (RASCAL study)[20], with 2721 patient samples collected from 13 different nations, indicated that the presence of a *KRAS* mutation increased the risk of recurrence and death, especially in a guanine (G) to thymine (T) mutation. Moreover, the expanded RASCAL Ⅱ study suggested that the prognostic role of the *KRAS* mutation, limited only to a glycine to valine mutation, was found in 8.6% of all patients and had a statistically significant effect on failure-free survival [P = 0.004, hazard ratio (HR) 1.3] and overall survival (OS) (P = 0.008, HR 1.29)[21]. However, in a translational study of PETACC3[22], a randomized phase Ⅲ trial showed that the *KRAS* mutation status does not have major prognostic value in stages Ⅱ and Ⅲ colon cancer. The difference in results may be largely due to the difference in sample size. The results from other trials are also not consistant[23].

KRAS as a predictive factor

Because *KRAS* is the most frequently mutated factor downstream of the EGFR signaling pathway, it was considered a candidate molecular biomarker for anti-EGFR therapy. In 2006, for the first time, the predictive value of *KRAS* was validated in a study by Lièvre *et al*[24] in which the *KRAS*-mutated patients showed no response to cetuximab and had a poorer OS compared with the wild-type *KRAS* patients. Later, a series of single-arm studies confirmed this result[25-29]. Then, not only cetuximab but also panitumumab were demonstrated to only be effective for wild-type *KRAS* patients[30,31]. These trials demonstrated that the outcomes of patients with wild-type *KRAS* were clearly better than those of the *KRAS*-mutant patients, although these were all retrospective analyses. The publication of two large, multicenter, randomized phase Ⅲ clinical trials unequivocally demonstrated the predictive value of *KRAS* for anti-EGFR therapy (Table 1). In these two trials, panitumumab or cetuximab *vs* best supportive care (BSC) was given to patients with chemorefractory CRC compared with BSC alone. Amado *et al*[10] demonstrated that the response rate of panitumumab was 17% and 0% for the wild-type *KRAS* group and the mutant group, respectively (P < 0.0001). In addition, when combined with chemotherapy [5-fluorouracil, leucovorin and irinotecan (FOLFIRI) or 5-fluorouracil, leucovorin and oxaliplatin], anti-EGFR

Table 1 Predictive value of *KRAS* for anti-epidermal growth factor receptor therapy in metastatic colorectal cancer

Reference	Regimen	Treatment line	Phase	*n*	Mutation status (%)	Method	Remarkable results
Monotherapy							
Karapetis *et al*[9], 2008	Cetuximab *vs* BSC	Chemotherapy refractory	III	394	42.3	Sequencing	Cetuximab alone works on patient with WT KRAS tumors
Amado *et al*[10], 2008	Panitumumab *vs* BSC	Chemotherapy refractory	III	427	43	Allele-specific PCR (DxS, United Kingdom)	Panitumumab alone works on patient with WT KRAS tumors
Combined with chemotherapy							
Van Cutsem *et al*[11], 2009	Cetuximab + FOLFIRI, FOLFIRI	First-line CRYSTAL trial	III	540	35.6	PCR clamping and HRM (TIB MolBioL, Germany)	Cetuximab plus FOLFIRI, reduced the risk of progression of metastatic colorectal cancer
Bokemeyer *et al*[12], 2009	Cetuximab + FOLFOX, FOLFOX	First-line, OPUS trial	II	233	42	PCR clamping and HRM (TIB MolBioL, Germany)	Significantly increased ORR in patients with WT KRAS tumors
Peeters *et al*[13], 2010	Panitumumab + FOLFIRI FOLFIRI	Second-line	III	1083	45	Allele-specific PCR (DxS, United Kingdom)	Significantly improved PFS in patients with WT KRAS tumors
Douillard *et al*[14], 2010	Panitumumab + FOLFOX FOLFOX	First-line	III	1096	40	Allele-specific PCR (DxS, United Kingdom)	Significantly improved PFS in patients with WT KRAS tumors
Van Cutsem *et al*[15], 2011	Cetuximab + FOLFIRI, FOLFIRI	First-line	III	1063	37	PCR clamping and HRM (TIB MolBioL, Germany)	Significantly improved OS in patients with WT KRAS tumors

BSC: Best supportive care; WT: Wild type; ORR: Overall response rate; FOLFIRI: 5-fluorouracil, leucovorin and irinotecan; FOLFOX: 5-fluorouracil, leucovorin and oxaliplatin; PFS: Progression-free survival; OS: Overall survival; PCR: Polymerase chain reaction; HRM: High-resolution melting.

antibodies (cetuximab or panitumumab)-treated patients had a better response rate and progression-free survival (PFS) or OS alone in the wild-type *KRAS* group, regardless of the treatment line[11-15]. Recently, better OS (median, 23.5 mo *vs* 20.0 mo; HR 0.796, $P = 0.0093$) was found in the cetuximab plus FOLFIRI-treated wild-type *KRAS* patients compared with the FOLFIRI-treated KRAS-mutated patients[15]. According to a recent meta-analysis of 11 studies conducted between 1966 and 2010[32], the *KRAS* status and the adding of anti-EGFR antibodies to standard chemotherapy were closely related to PFS [95% confidence interval (CI): 57%-90%, $P = 0.005$] and response rate (95% CI: 8.22%, $P < 0.001$).

On the basis of these results, National Comprehensive Cancer Network (NCCN), American Society of Clinical Oncology (ASCO) and European Medicines Evaluation Agency recommended testing for *KRAS* gene mutations in advanced CRC patients. The NCCN added *KRAS* testing to their 2009 clinical practice guidelines for colon and rectal cancers[33,34] and stipulated that only patients with wild-type (normal) *KRAS* genes should receive treatment with cetuximab (Erbitux) or panitumumab (Vectibix). The ASCO, in the same year, proposed a provisional clinical opinion (PCO)[35] demonstrating that testing for *KRAS* mutations should be performed prior to anti-EGFR monoclonal antibody therapy and that patients with KRAS mutations in either codon 12 or 13 should not receive this therapy as part of their treatment. This recommendation is slightly different from the NCCN guideline because the use of anti-EGFR therapy in the *KRAS*-mutated patients may be toxic.

KRAS TESTING STATUS

Frequency of testing

In a recent three cross-sectional survey performed in Europe, Latin America and Asia[36], physicians completed questionnaires on four patients per year. An analysis of 3800 samples per year showed that the *KRAS* testing frequency in metastatic CRC patients increased from 3% in 2008 to 47% in 2009 and 69% in 2010. It appears that the importance of *KRAS* mutation testing has become progressively understood by physicians and oncologists. Because implementation of the testing in the clinical practice has begun, it is essential to identify testing performance, as there are no set criteria for the process of *KRAS* detection, i.e., the selection of tissue specimens, specimen preparation, the timing of testing and the best method.

External quality assessment

A *KRAS* external quality assessment protocol was established in 59 laboratories throughout eight different European countries[37]. In the first assessment round, the results were unsatisfactory. The samples, including unstained sections of 10 invasive CRC with a known *KRAS* mutation status, were tested by each laboratory using their own preferred method for histological evaluation, DNA isolation, and mutation analysis. The test results were centrally validated by one of two reference laboratories. Only 70% of the laboratories correctly identified the *KRAS* mutational status in all samples, and the reports often lacked essential information. In another quality assessment for *KRAS* testing in Italy, five CRC specimens with known *KRAS* mutations were sent to be tested in 59 centers[38]. The limit to pass the assessment was set at 100% true responses. Only two centers failed in both the first round and the second round of testing. In Canada, until recently, there has been no such quality assessment. However, a guideline was developed according to a Canadian consensus conference held in Montreal in April 2010, in which the expert group provided recommenda-

tions on *KRAS* testing in the treatment of CRC[39]. In the United States, there is currently no FDA-approved standardized test. However, the PCO provided recommendations to the *KRAS* testing clinics. In Asia, there has been no external quality assessment system as yet, and it is critical to fulfill this objective.

Mutation status

As reported in 2011, the *KRAS* mutation frequencies in Asia, Europe, Latin American were 24%, 36% and 40%, respectively ($P < 0.0001$)[36]. It is unclear why a lower incidence is observed in Asian patients. In China, *KRAS* mutations were detected in 33.3% (30/90) of the CRC tumor samples using the nucleotide sequence analysis method[40]. These results significantly correlated with the response rate and survival time of cetuximab-treated patients. The difference of mutation status may result from many aspects, such as the tissue, the percent of tumor cells, the extracted DNA quality, the testing methods and the testing target.

Testing target

Currently, in most of the *KRAS* detection methods, only mutations of codon 12 or 13 are certified as informative for selecting non-responders to the anti-EGFR treatment in large clinical trials[15]. Therefore, mutation analysis of these sites is recommended. However, recent research has revealed new findings. Mutations in exons 3 and 4 are also effective in predicting the efficacy of EGFR-antibodies[41,42]. Codon 61 was found to account for 2% of all *KRAS* mutations and, similar to some of the codon 12 mutations, had predictive value[43]. Therefore, codon 61 may be useful in *KRAS* mutation testing. In contrast, not all mutations in codon 13 appear to be informative. In a recent analysis, cetuximab surprisingly worked on patients with chemotherapy-refractory CRC with p.G13D-mutated tumors, and these patients have a longer overall and PFS compared with those with the *KRAS*-mutated tumors[44]. Therefore, efforts are still required to confirm the importance of various mutations of the *KRAS* gene.

Sample selection

The most widely used tissue for *KRAS* testing is formalin-fixed paraffin-embedded (FFPE) tissue blocks[45], which are easy to obtain and convenient to preserve. However, DNA extracted from FFPE is time consuming and may be of poor quality, which can also result in false-positive or false-negative results due to an incomplete tissue fixation or tissue overfixation. Another specimen type is frozen tissue. Studies that compared the mutation detection rates in frozen and FFPE samples from the same tissue have found that the mutation rate in frozen samples is higher than that detected in FFPE samples[46]. The use of frozen tissue is suggested to be the gold standard for analysis, but the associated expense and technical difficulty of using frozen tissue make this method unsuitable for routine testing. In contrast, a high concordance was observed between primary tumors and metastatic locations (91.7%-96.4%)[47-49]. Therefore, the *KRAS* status in a primary site can be used for selecting patients who would benefit from anti-EGFR therapy. However, *KRAS* status can be heterogeneous within a primary tumor, and thus, different parts of such tumors should be examined to accurately predict the *KRAS* status in metastatic lesions.

Beyond the selection of tissue, other choices, such as peripheral blood, have been studied. Yen *et al*[50] detected circulating tumor cells with *KRAS* oncogenes using membrane arrays; *KRAS* mutations were identified in 39.5% (30/76) of peripheral blood samples, which is similar to that in tumors (43.4%). According to a review concerning the validation of *KRAS* mutation testing in CRC blood samples which summarizes the studies that detect *KRAS* status using tissue or plasma/serum[51], a positive *KRAS* mutation in plasma or serum suggests a *KRAS* mutation in the tumor whereas the absence of a *KRAS* mutation in the plasma or serum does not necessarily prove a lack of a similar mutation in the CRC tumor tissue. Further studies are needed in this field.

Methods

A number of methods can be used in *KRAS* mutation testing, with different sensitivity, turnaround time, and cost. In the NCCN guideline or ASCO PCO, no explicit method was assigned. Therefore, the use of assays worldwide is somewhat chaotic. In the Italian quality assessment for *KRAS* testing[38], five CRC specimens were sent to 59 centers, which were asked to use their own preferred method for DNA extraction and mutational analysis. Of these 59 centers, polymerase chain reaction (PCR) sequencing was the predominant method for mutational analysis, as 48 (81.3%) centers used this methodology. Among the remaining centers, 5 centers (8.5%) used pyrosequencing, 3 centers (5.1%) used Real-Time PCR (Therascreen kit), 2 centers (3.4%) used restriction fragment length polymorphism (RFLP) analysis and 1 center (1.7%) used the KRAS strip assay. In the United States, the amplification refractory mutation system was used by most laboratories[52].

The traditional methods used for mutation testing are hybridization and DNA sequencing. These methods are complex and time consuming. The emergence of polymerase chain reaction (PCR) sheds new light on this field. Currently, mutation testing methods are almost exclusively based on this technology, including PCR-based sequencing, high resolution melting analysis (HRMA), amplification refractory mutation system (ARMS), and cleaved amplification polymorphism sequence-tagged sites (PCR-RFLP).

Among these methods, DNA sequencing, also called Sanger sequencing or dideoxy sequencing, is considered the gold standard because this methodology analyzes the DNA sequence nucleotide by nucleotide and can identify all possible mutations in the analyzed *KRAS* gene segment, including base substitutions, insertions and deletions. However, this approach has a low sensitivity of about 20%, and is laborious and time consuming. An alternative approach to this methodology, i.e.,

Table 2 Methods used for *KRAS* mutation testing[45,55,60-62]

Method	Sensitivity (mutant/wild-type) (%)	Turnaround time	Main advantages	Main disadvantages
Sanger sequencing	20–30	Slow (4 d to 2 wk)	Detects all possible mutations, cost-effective	Insensitive, time consuming, open PCR system is easily contaminated
Pyrosequencing	5	Rapid	Detects all possible mutations, sensitive	Open PCR system is easily contaminated
Real-time PCR with HRMA	5	Rapid	Rapid, closed PCR system, detects all possible mutations (heterozygous and homozygous)	Occasionally difficult to distinguish between mutation types
Allele-specific real-time PCR	10	Rapid	Rapid, closed PCR system	Detects only the 7 most common mutations, requires more tissue for analysis compared with other methods
RFLP with sequencing	0.1	Slow (4 d to 2 wk)	Sensitive	Requires confirmation by sequencing, complicated
DxS (ARMS/S)	1	Rapid	Sensitive, time-saving	Expensive, detects specific mutations targeted by the designed primers
COLD-PCR with sequencing	1-2.5	Rapid	Sensitive, cost-effective, detects all possible mutations	-

ARMS: Amplification refractory mutation system; RFLP: Restriction fragment length polymorphism; PCR: Polymerase chain reaction; COLD-PCR: Coamplification at lower denaturation temperature PCR.

pyrosequencing, has a sensitivity proven to be approximately 5%-10% and has commercialized the detection of *KRAS* mutations; corresponding commercial kits, the PyroMark® (Qiagen, Valencia, CA, United States), have been developed[53,54].

Of the non-sequencing methods, ARMS[55], real-time PCR analysis with HRMA[56], RFLP[57] and allele-specific real-time PCR[58], most of which are based on real-time PCR technology, have been well studied in the past three years with Sanger sequencing as the reference, demonstrating the effectiveness and availability of these methods for *KRAS* status testing. A multicenter study[59], which evaluated six different *KRAS* mutation detection methods, including pyrosequencing, HRMA, dideoxy sequencing, and two commercial kits, showed a concordant *KRAS* status in 66/80 (83%) of frozen tissue samples and 71/74 (96%) of paraffin tissues using the five best performing assays. Each of the assays has its advantage and limitations, and as details have been described in previous publications, we have summarized some notable features in Table 2[45,55,60-62]. The HRMA assay, often based on real-time PCR, detects the mutant sequence through measuring changes in the melting of a DNA duplex with the aid of intercalating dyes. This method is fast and sensitive but has been reported to have a false-positive rate of 20%[63]. Therefore, this method requires sequencing confirmation and cannot show the concrete mutation pattern. The allele-specific Real-Time PCR and ARMS can only detect the limited mutation sites of the *KRAS* gene, which makes these methods less feasible in clinical practice. The ARMS-based commercial kit, TheraScreen® (DxS Ltd, Manchester, United Kingdom), however, has been widely used in laboratories[64]. This kit has a real-time PCR-based assay that combines the ARMS with Scorpion probes (seven probes for seven different mutations in *KRAS*), eliminating the need for post-PCR confirmation by direct sequencing, and is thought to be the most sensitive method until recently with a sensitivity of 1%[45].

Recently, more sensitive methods have been utilized in *KRAS* detection. One method is the PCR-clamp assay, and the other is coamplification at lower denaturation temperature PCR (COLD-PCR). The PCR-clamp assay utilizes mutation-specific hybridization probes and another wild-type-complementary peptide nucleic acid probe to suppress the amplification of the normal sequence and can detect less than 1% of the allele[65,66]. A commercial kit (KRAS LightMix) by TIB MolBiol (Berlin) uses this technology and a melting curve analysis and has been used in multicenter, phase Ⅲ clinical trials in which patients were treated with the anti-EGFR antibody, cetuximab[11,12,15]. COLD-PCR is another selective amplifying system that enriches the "minority alleles" from the mixed DNA sequences based on the lower melting temperature of mutant homoduplexes as compared with wild-type ones. Therefore, in COLD-PCR, the denaturation temperature is set at 80 ℃ whereas the denaturation temperature in conventional PCR is approximately 94 ℃. Using this principle, this technology does not require special equipment or reagents or time-consuming procedures. As a sensitive DNA enrichment method, COLD-PCR is often followed with HRMA or pyrosequencing. Mancini *et al*[67] demonstrated that COLD-PCR combined with HRM can improve the limit of detection of *KRAS* and *BRAF* mutations in CRC, increasing the percentage of mutated CRCs from 40% (47/117) to 48.7% (57/117) compared with traditional PCR and direct sequencing. In another study by Zuo *et al*[68], COLD-PCR combined with pyrosequencing detected all the mutations in 50 samples, including DNA extracted from either fresh or FFPE tissue specimens that were confirmed positive by conventional PCR, and the mutation detection sensitivity was certified as 1.5%.

In addition, COLD-PCR combined with HRMA

assay does not require expensive and time-consuming procedures; thus, in clinical settings, this procedure has the potential to be used to select those patients who are eligible for EGFR-targeted therapies.

Our recommendation

Currently, it is accepted that the DNA fragmentation caused by improper fixation, heterogeneous somatic *KRAS* gene mutations, and the influence of stromal cells can cause false-positive *KRAS* mutation testing results. Fortunately, the technique refinements and sufficient tissue selected can reduce this limitation. It is suggested that at least 300 tumor cells or 30 ng of template DNA are required for *KRAS* status analysis. However, the appropriate method to extend to the clinic is still unclear. Molinari *et al*[69] found that highly sensitive methods could improve the accuracy of predictions of anti-EGFR monoclonal antibody efficacy. Therefore, assay sensitivity when detecting *KRAS* mutations is a key issue for correctly analyzing tumor specimens. However, Carotenuto *et al*[70] demonstrated that in samples with more than 30% tumor cells, the DxS assay and PCR-sequencing, which are the most sensitive and non-sensitive methods, respectively, showed no difference in identifying *KRAS* mutations. Therefore, more effective and sensitive methods are required for inconclusive samples and those with a low number of tumor cells. Upon considering the sensitive detection methods, as previously described, pyrosequencing is a new, robust but expensive technology. The DxS assay (ARMS/S) is now widely used in clinical labs but can only detect the seven common mutations, and it is costly. COLD-PCR, which can enrich the mutant alleles, is considered a simple method that increases *KRAS* testing sensitivity. Therefore, we recommend the use of this assay combined with HRM or sequencing for determining *KRAS* status; although, this approach should be validated by further large sample studies.

CONCOMITANT ANALYSIS WITH OTHER FACTORS

Unfortunately, *KRAS* mutations account for approximately 35% of the nonresponsive patients that receive anti-EGFR treatment[35]. Therefore, using *KRAS* as a predictor of clinical outcomes is not always useful. These results have led researchers back to the molecular mechanisms of cetuximab and panitumumab resistance to find other powerful prognostic markers. *BRAF*, which is another member of EGFR signaling cascade, is located downstream of *KRAS* and is considered the most promising marker for predicting anti-EGFR treatment resistance apart from *KRAS* gene. *BRAF* mutations mainly occur at exon 15 with a frequency of approximately 5% to 10% and the common V600E pattern. It is notable that *BRAF* and *KRAS* mutations are mutually exclusive ($P < 10^{-6}$)[71]. Therefore, *BRAF* mutation analysis is recommended when the *KRAS* gene is the wild type. Di Nicolantonio *et al*[72] found in a retrospective study that none of the *BRAF*-mutated patients responded to cetuximab or panitumumab and that none of the responders carried *BRAF* mutations ($P = 0.029$). In addition, *BRAF*-mutated patients had a significantly shorter PFS ($P = 0.011$) and OS ($P < 0.0001$) compared with wild-type patients. On the basis of these results, the NCCN clinical guidelines in 2010 currently recommend *BRAF* mutational status assessment of metastatic CRC patients with a wild-type *KRAS* to guide the therapeutic use of cetuximab and panitumumab.

Apart from the *KRAS* and *BRAF* gene mutations, other genetic aberrations, such as *PIK3CA* and *PTEN*, were demonstrated to be helpful in predicting the resistance to anti-EGFR treatment[40,73]. In addition, many oncologists and pathologists have proposed that combining the analysis of these factors simultaneously will provide a clearer overall prognostic indication for EGFR inhibitor status. The recent data from a retrospective analysis demonstrated that when the loss of *PTEN* expression and mutations of *KRAS*, *BRAF* and *PIK3CA* are concomitantly ascertained, as many as 70% of the metastatic CRC patients can be identified as unlikely to respond to anti-EGFR therapies[74]. Therefore, CRCs lacking alterations in *KRAS*, *BRAF*, *PTEN* and *PIK3CA*, which may have the highest probability of response to anti-EGFR therapies, are defined as "quadruple negative"[74,75].

In addition, in a retrospective consortium analysis[43], the largest series to date according to our knowledge, the effects of *KRAS*, *BRAF*, *NRAS* and *PIK3CA* mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal was studied. In total, 1022 tumor DNA samples were tested, of which 40.0% (299/747) harbored a *KRAS* mutation, 14.5% had a *PIK3CA* mutation, 4.7% had a *BRAF* mutation, and 2.64% *NRAS* mutation, and carriers of the four mutations had a lower response rate to the cetuximab plus chemotherapy treatment compared with those lacking any of the four mutations. A multivariate analysis also confirmed that if *KRAS* is unmutated, assessing the *BRAF*, *NRAS*, and *PIK3CA* exon 20 mutations provides additional information about patient outcomes. It is notable that while *NRAS* accounts for only 2.64% of these molecular alterations, this mutation is associated with unresponsiveness to panitumumab treatment.

It is obvious that *KRAS* mutational status analysis is insufficient for predicting the efficacy of anti-EGFR therapy, and adding the concomitant analysis of downstream factors can be helpful in selecting the correct patient for this personal treatment. In addition, we suggest that microsatellite inestability (MSI) be added to this concomitant analysis.

Microsatellite instability, defined as small deletions or expansions within short tandem repeats in tumor DNA resulted from the inactivation of the DNA mismatch repair system, has been found in up to 90% of the tumors of the hereditary nonpolyposis CRC and in approximately 20% of sporadic colorectal tumors[76,77]. Using a panel of 5 microsatellites recommended by the National

Cancer Institute, i.e., BAT 25 and BAT 26 (mononucleotide repeats), D2S123, D5S346 and D17S250 (dinucleotide repeats), CRC tumors are classified as MSI-high (MSI-H), MSI-low (MSI-L) and microsatellite stability (MSS), and the MSI-H was thought to indicate a more favorable prognosis[78]. However, with regard to predicting therapy response, the role of MSI is conflicting. Recently, some researchers have combined *KRAS* and MSI in their study[79] and found that both genes are prognostic of CRC. In another study[80], the combined analysis of specific *KRAS* and *BRAF* mutations, and microsatellite instability were used to identify prognostic subgroups of sporadic and hereditary CRC. As the result, 3 distinct prognostic subgroups were observed in univariate (P = 0.006) and multivariable (P = 0.051) analysis: group 1 consisted of patients with *KRAS G12D* or *G12V* or *BRAFV600E* mutations independent of MSI status; they had a poor survival time and suffered more patient deaths. Group 2 included patients with either wild-type *KRAS*/*BRAFV600E* or *KRAS* G13D mutations in the MSS/MSI-L tumors and had a more favorable outcome. Finally, the patients with MSI-H cancers and simultaneous *G13D* mutations were observed to have the worst outcomes. The survival times for groups 1-3 varied significantly (P = 0.006). Therefore, we recommend the concomitant analysis of *KRAS, BRAF, PIK3CA,* and *PTEN* combined with MSI, which can facilitate selecting the appropriate patients for anti-EGFR treatment while also indicating the outcome of CRC patients.

CONCLUSION

KRAS, an important member of the EGFR signaling cascade, can acquire activating mutations in codons 12 and 13 of exon 2 in approximately 35%-45% of the CRC cases, rendering EGFR inhibitors ineffective. Though the prognostic value of *KRAS* is conflicting, it is a promising predictive biomarker of personalized treatment. Numerous clinical trials have clarified the significant benefit of outcomes in patients with wild-type *KRAS* for anti-EGFR therapy, despite the treatment line. Therefore, *KRAS* status testing has been recommended by national organizations, including NCCN, American Society for Clinical Oncology and European Medicines Agency. In recent years, *KRAS* testing is administered with a high frequency; however, standards are desired worldwide, including the selection and processing of the tumor sample and the choice of the appropriate detection method, which may affect the accuracy of the testing results. COLD-PCR is a simple assay that can increase *KRAS* testing sensitivity by enriching the mutant alleles. This technology combined with HRM or sequencing is potentially useful in *KRAS* detection in a clinic practice. In addition, concomitant analysis with other factors, such as BRAF, PIK3CA, PTEN and MSI, is helpful in supporting *KRAS* as predictive and prognostic factors, but further efforts are needed prior to implementation.

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ORIGINAL ARTICLE

Effect of double-balloon enteroscopy on pancreas: An experimental porcine model

Rafael Latorre, Federico Soria, Octavio López-Albors, Ricardo Sarriá, Francisco Sánchez-Margallo, Pilar Esteban, Fernando Carballo, Enrique Pérez-Cuadrado

Rafael Latorre, Octavio López-Albors, Ricardo Sarriá, Department of Anatomy and Comparative Pathology, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain
Federico Soria, Francisco Sánchez-Margallo, Jesús Usón Minimally Invasive Surgery Center, 10071 Cáceres, Spain
Pilar Esteban, Enrique Pérez-Cuadrado, Digestive Service, Morales Meseguer Hospital, 30008 Murcia, Spain
Fernando Carballo, Digestive Service, Virgen de la Arrixaca Hospital, 30120 Murcia, Spain
Author contributions: Latorre R, Soria F, López-Albors O, Sarriá R directed the study, including substantial contributions to conception, acquisition of data, analysis and interpretation of data, and manuscript writing; Pérez-Cuadrado E designed the study and wrote the manuscript including drafting the article, and critically revising it for important intellectual content; Esteban P, Carballo F and Sánchez-Margallo F analyzed the data.
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Correspondence to: Rafael Latorre, PhD, Department of Anatomy and Comparative Pathology, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain. latorre@um.es
Telephone: +34-868-884697 Fax: +34-868-884147
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Abstract

AIM: To evaluate the effect of double-balloon enteroscopy (DBE) on pancreas histology and levels of pancreatic enzymes.

METHODS: Conventional upper gastrointestinal endoscopy was performed on five control pigs. Oral DBE was performed with an EN-450T5 enteroscope on 20 pigs. Two experimental groups (10 pigs each) were defined according to DBE duration: 90 min for Group 1 and 140 min for Group 2. During oral insertion, the balloons were not inflated in the descending part of the duodenum to avoid the minor duodenal papilla. Serum amylase, lipase and C-reactive protein (CRP) levels were monitored before the procedure and repeated every 30 min until the exploration was finished, as well as 24 h and 7 d after. After the procedure and for a total of 7 d, the pigs were observed twice a day for signs of decreased activity, irritability, vomiting or anorexia. Gross and microscopic examination of the pancreas was performed on day 7.

RESULTS: All animals tolerated DBE without clinical manifestations of acute pancreatitis. Experimental groups had higher levels of enzymes than the control group at 24 h. Throughout the exploration, the amylase levels increased significantly above the baseline 24 h after DBE, although the increase was not statistically significant and did not reach 20% of the baseline. An increase in lipase and CRP was observed at 24 h after the procedure, although by day 7, all enzymatic levels had returned to baseline. No differences between Groups 1 and 2 were found for any enzyme and sampling site during and after the procedure. Similarly, no correlation between insertion depth and enzyme levels was observed. Direct *in situ* and post-removal inspection of the pancreas did not show any evidence of fluid collection, abscesses or hemorrhage. Histological examination of the pancreas from Groups 1 and 2 revealed the existence of focal areas (0.14-0.26 mm^2) of ischemic necrosis in 47.4% of the animals. In the pigs with damaged pancreas, the left lobe (tail) was always affected. However, this only happened in 83.3% of the samples from the right lobe (head) and in 33.3% of the samples from the body of the pancreas. Significant differences were found between the left lobe (tail) and the body for the percentage of affected pancreas. Both the size of the lesions and the percentage of affected pancreas were higher in the left pancreatic lobe (tail). The presence of the lesions was not related to the exploration length.

CONCLUSION: The increase in pancreatic enzymes

after DBE could be related to focal points of pancreatic ischemic necrosis due to mechanical stress.

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Key words: Endoscopy; Pancreatitis; Double-balloon enteroscopy; Experimental study; Pig model

Peer reviewer: Jan Bureš, MD, PhD, Professor, Second Department of Medicine Gastroenterology, Charles University in Praha, Faculty of Medicine at Hradec Králové, University Teaching Hospital, Sokolská 581, 50005 Hradec Králové, Czech Republic

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INTRODUCTION

Double-balloon enteroscopy (DBE) has enabled endoscopic diagnosis and treatment in the small intestine, which had been very difficult for many years[1]. Yamamoto *et al*[2] introduced DBE in 2001 and its usefulness is already recognized in many countries. DBE is considered a well-tolerated and safe endoscopic technique[3-8], but an increase in pancreatic enzymes and potential pancreatitis are recognized as complications directly attributed to the procedure[1,7,9-12].

The mechanism for post-DBE pancreatitis remains unclear. Potential explanations might include: (1) pancreatic duct obstruction by direct oppression of the papilla with the inflated balloon[7,9]; (2) reflux of intestinal fluid into the pancreatic duct owing to an increase in intraduodenal pressure because of mechanical strain[12,13]; or (3) prolonged mechanical injury or ischemia on the pancreas as a result of repeated stretching and shortening of the endoscope and overtube[7,14-16].

Unfortunately, levels of pancreatic enzymes several days after the procedure and evaluation of potential lesions in the pancreas under normal clinical conditions are unknown in humans. Also, to the best of our knowledge, there have been no studies in animal models to clarify the etiology of pancreatic hyperamylasemia and pancreatitis post-DBE. This study was aimed at determining the effects of the DBE technique on the pancreatic enzymes and histology under nonpathological conditions. To assess if the timing of DBE influences the pancreatic enzyme markers, two experimental groups with different DBE duration (90 or 140 min) were established.

MATERIALS AND METHODS

Animals and protocols

Twenty-five Large White pigs (35-40 kg) were used. The day before DBE, animals were fasted, with no liquid restrictions, and given a laxative preparation. Animals were prepared and anesthetized for the endoscopic procedures. Intravenous saline solution was administrated to secure basic hydration during the DBE procedure. After 24 h fasting, each pig was intramuscularly premedicated with diazepam 0.1 mg/kg, ketamine 10 mg/kg and atropine 0.01 mg/kg. General anesthesia was induced with propofol 2 mg/kg intravenously and maintained with sevofluorane 1.8%-2% delivered *via* an endotracheal tube. Animals from the control group (n = 5) underwent conventional upper gastrointestinal (GI) endoscopy. In the remaining 20 pigs DBE was performed with an EN-450T5 enteroscope (Fujinon, Japan) by experienced endoscopists. The exploration depth was estimated according to the methodology established by May *et al*[17].

Two experimental groups (10 pigs each) were defined according to DBE duration: 90 min for Group 1 and 140 min for Group 2. During the oral insertion of the scope and overtube, the balloons were not inflated in the descending part of the duodenum to avoid the major and minor duodenal papilla. Blood samples were taken before the procedure and during the exploration at 20 (control group only) 30, 60, 90 and 140 (Group 2 only) min, and also at 24 h and 7 d after DBE (Groups 1 and 2) and 24 h and 7 d after GI endoscopy (control group) to evaluate the serum concentrations of amylase, lipase and C-reactive protein (CRP). Animals were allowed to feed 24 h after DBE. After the procedure and for a total of 7 d, the pigs were observed twice a day for signs of decreased activity, irritability, vomiting or anorexia. On day 7, all the animals were euthanized with a pentobarbital overdose and the pancreases were removed. Each pancreas was examined *in situ*, palpated, removed from the cadaver and then sectioned to identify gross alterations. The right lobe (head) and left lobe (tail), as well as the body of the pancreas were preserved in 10% buffered formalin, trimmed into 1 cm × 1 cm × 1 cm tissue blocks (18-22 blocks per pancreas) and processed for histopathology after hematoxylin and eosin staining. Histology sections were studied under light microscopy (three fields per block of tissue) and when lesions were observed, the cross-sectional areas were measured with the SigmaScan Pro 5.0 program (Systat Software Inc., San Jose, CA, United States).

Ethical approval

All animals received humane care in compliance with the European Communities Council Directive (86/609/EEC). Protocols were approved by the local government Ethics Committee for Animal Research. The endoscopic equipment used was for research with animals only.

Statistical analysis

Data of enzyme levels were included in a spreadsheet and analyzed with SPSS 17.0 (SPSS, Chicago, IL, United States). Descriptive statistics were obtained and all the

Table 1 Comparison of serum enzyme levels between double-balloon enteroscopy (Group 1) and control group at different sampling intervals

	Amylase (U/L)	Lipase (U/L)	CRP (U/L)
t_0			
Control	1295.74 ± 120.55	14.66 ± 2.16	11.02 ± 3.92
DBE	2074.42 ± 296.82	17.74 ± 2.44	39.14 ± 13.02
t_{end}			
Control	1290.62 ± 158.58	9.38 ± 1.02	11.86 ± 3.8
DBE	2070.99 ± 281.31	8.84 ± 1.15	37.16 ± 12.1
$t_{24\ h}$			
Control	1311.02 ± 88.56	19.48 ± 3.6	51.46 ± 26.02
DBE	2487.18 ± 364.46[b]	26.73 ± 6.63	114.81 ± 31.84
$t_{168\ h}$			
Control	1474.42 ± 143.16	8.86 ± 2.21	20.36 ± 10.52
DBE	2337.9 ± 300.72	5.71 ± 0.51	78.07 ± 31.6

t_0: Samples before the procedure; t_{end}: Samples at the end of the procedure [gastrointestinal endocopy (GI) in control group and double-balloon enteroscopy (DBE) in experimental group]; $t_{24\ h}$: Samples 24 h after the procedure (GI in control group and DBE in experimental group); t_{168h}: Samples 7 d after the procedure (GI in control group and DBE in experimental group). [b]$P < 0.01$ *vs* control group.

variables tested for normality (Kolmogorov-Smirnov test) before being subjected to analysis of variance (ANOVA) (linear model with repeated measures). Within-subject factors were the different timing of blood sampling and the inter-subject factor was the duration of the exploration (90 or 140 min). Tukey and Bonferroni tests were used to ascertain *post hoc* differences. The possible association between the experimental groups and the presence of lesions in the pancreas was checked with the χ^2 test. In addition, the nonparametric Mann-Whitney test was used to ascertain any dependence between the size of the lesions and the portion of the pancreas.

RESULTS

Procedure evaluation

All the animals tolerated the procedure without any clinical manifestations of pancreatitis or distress. During the endoscopic exploration, passing the endoscope and the overtube into the duodenum was not difficult (< 3 min). Remnants of food in stomach did not make DBE more difficult. During the 7 d observation period after the procedure, the activity and dietary intake were normal in all the animals.

Estimations were calculated using the depth of insertion technique described by May *et al*[5]. The average insertion depth in Group 1 was lower than in Group 2: 268 cm (range: 209-336 cm) and 333 cm (range: 230-488 cm), respectively. Nevertheless, due to high data variability, the ANOVA for the insertion depth between the two groups was not significant ($P = 0.181$).

Biochemical evaluation

No statistical differences between sampling states in the control group were found. Experimental Group 1 had higher levels of enzymes than the control group at 24 h (Table 1). These differences were also present between the control group and Group 2.

Enzyme serum levels at the different sampling stages are displayed in Figure 1. To simplify notation, values at 30 and 60 min of the procedure are omitted because they were always similar to time 0.

All the animals had similar basal amylase levels before the procedure (approximately 2000 IU). Throughout the exploration, no significant changes in the amylase levels were noted (Figure 1A). However, the amylase levels increased significantly above the baseline 24 h after DBE, although the increase did not reach 20% of the baseline level. On day 7 after the procedure, the amylase level decreased progressively to the baseline, but it was still significantly higher in Group 1.

Lipase levels showed a variable trend during and after the exploration (Figure 1B). This is well illustrated in Group 1, where there was a significant decrease during the exploration, peak levels at 24 h after the procedure, and the lowest levels 7 d later.

CRP levels were significantly higher 24 h after DBE (more than twice the initial levels) (Figure 1C). However, CRP concentrations then decreased progressively towards the baseline, so no significant differences were found between the initial levels and 7 d after DBE.

No differences between Groups 1 and 2 were found for any enzyme and sampling site during and after the procedure. Similarly, no correlation between insertion depth and enzyme levels was observed (P for Pearson coefficient was always > 0.3).

Histology evaluation

Direct *in situ* and post-removal inspection of the pancreas did not show any evidence of fluid collection, abscesses or hemorrhage (Figure 2).

Light microscopy examination of tissue samples from the control group showed occasional small areas with infiltration and edema. However, the tissue samples from Groups 1 and 2 revealed the existence of limited areas of ischemic necrosis scattered throughout the parenchyma (Figure 3). This was observed in nine of the 19 pigs (47.4%). It should be noted that the histology samples from one pig were not included in the analysis due to bad processing. Some of the necrotic areas showed slight inflammatory cell infiltration around the sites of necrosis, but alterations to the pancreatic duct system were rare. In the nine pigs with damaged pancreas, the left lobe (tail) was always affected. However, this only happened in 83.3% of the samples from the right lobe (head) and in 33.3% of the samples from the body of the pancreas. The average area of lesions (μm^2) related to each portion was: right lobe (head): 198274 ± 23952, body: 136782 ± 24163 and left lobe (tail): 260516 ± 32819. The percentage (%) of affected pancreas in each portion was: right lobe (head): 1.83, body: 0.60 and left lobe (tail): 3.11. Although the overall differences were not statistically significant, the average values for both parameters were higher in the left

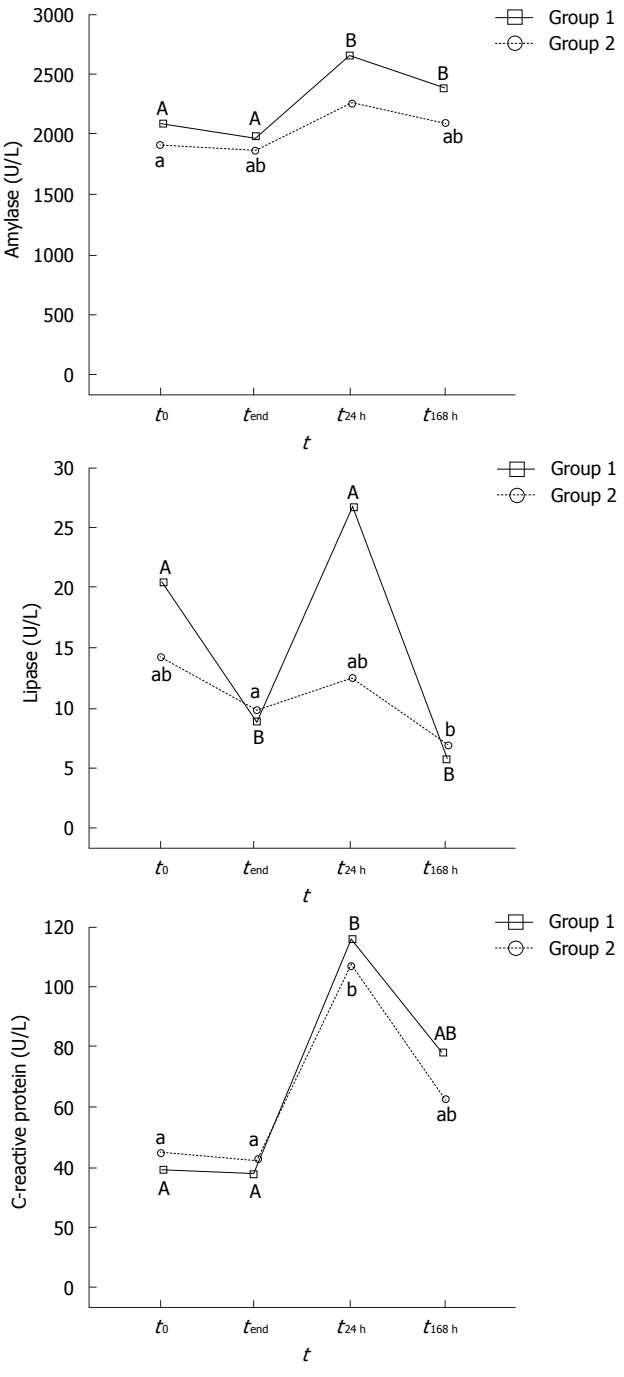


Figure 1 Plots representing the serum levels of amylase (A), lipase (B) and C-reactive protein (C) for Groups 1 and 2 at different sampling intervals. t_0: Samples before the procedure; t_{end}: Samples at the end of the procedure [gastrointestinal endocopy (GI) in control group and double-balloon enteroscopy (DBE) in experimental group]; $t_{24\ h}$: Samples 24 h after the procedure (GI in control group and DBE in experimental group); $t_{168\ h}$: Samples 7 d after the procedure (GI in control group and DBE in experimental group). Within-group differences: sampling stages with no coincident capital (Group 1) or normal case (Group 2) letters were significantly different ($P < 0.05$). No significantly different results between Groups 1 and 2 were found at any sampling stage.

pancreatic lobe (tail). Significant differences were found between the left lobe and the body for the percentage of affected pancreas. Interestingly, the presence of the lesions was not related to the exploration length (90 or 140 min; Pearson χ^2 and Fisher's exact tests > 0.3).

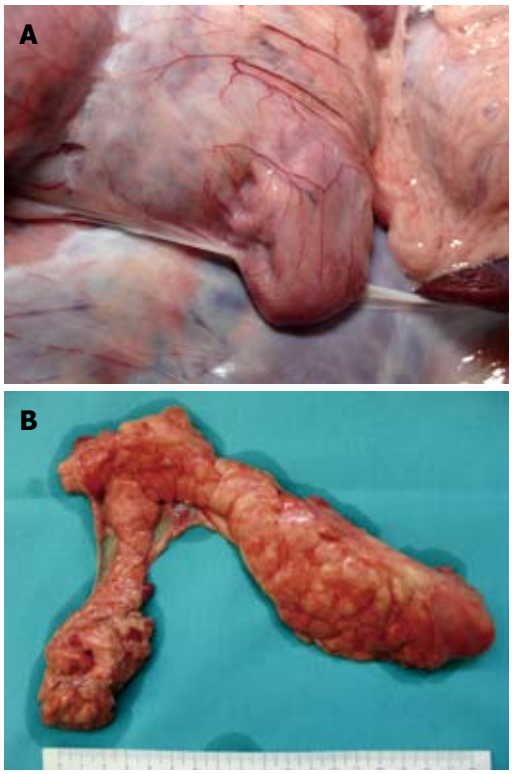


Figure 2 Gross anatomy of the porcine pancreas after double-balloon enteroscopy. A: *In situ* image of the left lobe (tail); B: Aspect of the whole pancreas immediately after removal from cadaver.

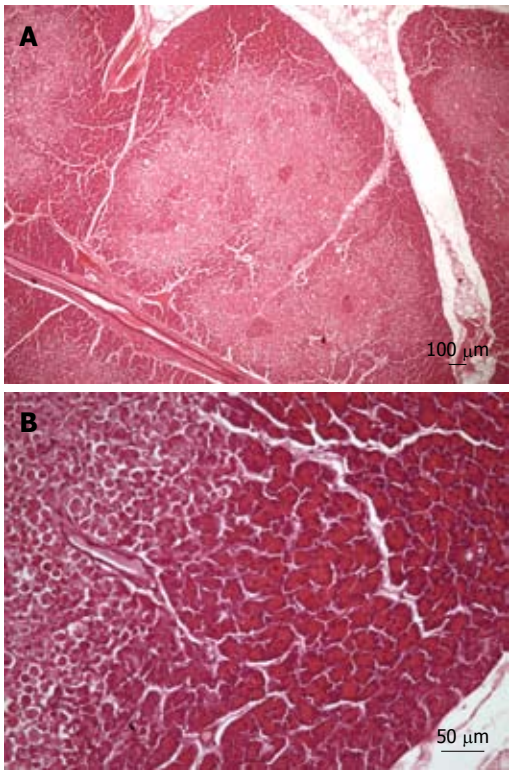


Figure 3 Light microscopy pictures of the porcine pancreas after double-balloon enteroscopy. A: Light microscopy of porcine pancreas after double-balloon enteroscopy showing located ischemic necrosis in pancreatic interlobular tissue; B: Magnification of previous image, view of margin between necrosis and viable tissue.

DISCUSSION

A low incidence (0.3%) of acute pancreatitis after diagnostic DBE has been reported in retrospective studies in Europe and Asia[18], and also in the United States[19,20]. However, latent hyperamylasemia without the development of pancreatitis occurs after peroral DBE more frequently than was previously thought[9,10,21].

The physiology and anatomy of the porcine pancreas is similar to that in humans, that is, it is a partially retroperitoneal organ and the pancreatic body wraps the portal vein. The firmness of the pancreatic parenchyma in swine is also similar to the gland in humans[22]. Thus, the porcine model has been used in many types of studies related to the pancreas such as endoscopic approaches to the pancreas[23-25], or experimental obstructive pancreatitis[26]. An *ex vivo* model for training has also been developed on porcine intestine[5,27]. Recently, the swine model has been validated for both DBE training and research, altogether improving the safety conditions of DBE in humans[28].

The characteristics, in terms of duration and insertion depth, of this study have been designed for comparison with DBE in humans. The DBE time length in Group 1 (90 min) and Group 2 (140 min) was selected in accordance with published average values from prospective studies in humans. Thus, Mehdizadeh *et al*[29] referred to a duration of 109.1 ± 44.6 min for the first 10 cases and 92.4 ± 37.6 min for subsequent cases. Similar times were referred to by other authors, namely, 75 min[14], 95 ± 42 min,[7] 115 ± 9 min[21], and 148 min[10,16]. Our results for the average insertion depth in both groups of animals were within the same range as those reported in previous works: 240 ± 100 cm[5], 250 ± 170 cm[30], 220 ± 90 cm[6], 270 ± 100 cm[7], and 351 ± 108 cm[21].

According to the literature, three authors have specifically measured amylase levels in patients before and after oral DBE[9,10,21]. Honda *et al*[9] found that 46% of patients undergoing DBE developed hyperamylasemia. Kopácová *et al*[10] investigated the levels of serum amylase, lipase and CRP both before and after DBE (4 h and 24 h). They found increased levels of amylase and lipase in 51.4% of the patients 24 h after the procedure. However, only 2.8% of them suffered acute pancreatitis. Pata *et al*[21] also checked levels of serum amylase and lipase both before and 4 h and 12 h after DBE. Just 4 h after the procedure, they found 25% of the patients had hyperamylasemia and hyperlipasemia, and 12.5% of the patients had pancreatitis. It is important to pay attention to the fact that the increases in the serum pancreatic amylase described in those three previous studies were twice or even three times higher than normal levels. In contrast, serum levels of amylase and lipase in the present work never reached twice the baseline level, and this could be related to the fact that the balloons were always inflated after the site of the pancreatic duct opening in the duodenum. In support of this, Pata *et al*[21] have described that amylase levels after DBE are negatively correlated to the depth at which the balloons are first inflated. Interestingly, no changes in the levels of serum amylase and lipase have been reported in patients when the first inflation of the balloons was done after reaching the ligament of Treitz[14,31,32]. On the other hand, some authors have described that substantial hyperamylasemia tended to be associated with longer duration of DBE[10,15,21,32]. However, we did not find such a difference, and the enzyme levels of Groups 1 and 2 did not vary significantly at any stage. Similar results have been reported by others authors[9,16].

The mechanical stress to the small intestine, mesentery and pancreas has also been suspected[7,9] to cause increased levels of amylase, lipase and CRP. Thus, a plausible explanation for the increased levels of enzymes[9,12,13] is an effect of the increased intraluminal pressure on the pancreatic ducts allowing intestinal fluid to progress towards the pancreas, and as such, should be kept in mind. However, the histological injuries found in this study are more likely related to an ischemic process in the vascular supply to the pancreas. The continuous pressure of the small intestine and the mesentery during the push and pull maneuvers could compromise the vascular supply to the pancreas, resulting in an increase in the pancreatic enzymes and unspecific inflammatory factors such as CRP. Along these lines, several works have reported pancreatic vascular restriction as a potential mechanism for hyperamylasemia after oral DBE[11,16,33]. On the other hand, the larger and more frequent areas of ischemic necrosis in the left lobe (tail) of the pig pancreas seem to be related to the particular vasculature of this pancreatic portion. In pigs, the left lobe (tail) is supplied by a single artery, which is a branch of the splenic artery. A similar situation is found in humans where the main artery of the tail of the pancreas is the major pancreatic artery. Such anatomical particularity predisposes the left pancreatic lobe to suffer from hypoxia or even ischemia if there is any mechanical restriction to the blood supply through this artery. Although this explanation requires further specific research, it is interesting to highlight that computed tomography has revealed that human pancreatitis is predominantly located in the tail of pancreas[15,34]. Considering the variance of the enzyme levels and that this was a non-survival animal model study, the number of animals could be a limitation of this work.

In conclusion, the inflation of the balloons after the duodenal papilla diminished the iatrogenic effects on the pancreas. However, minor enzymatic alterations and focal lesions in the pancreas remained, which on the other hand, failed to cause any clinical signs of pancreatitis. A vascular component is probably involved in the etiology of DBE-related pancreatic alterations, but this topic needs further research aimed at evaluating the effects of DBE exploration maneuvers on the vascular supply to the pancreas.

COMMENTS

Background

Double-balloon enteroscopy (DBE) has enabled endoscopic diagnosis and treatment in the small intestine, but an increase in pancreatic enzymes and

potential pancreatitis are recognized as complications directly attributed to the procedure.

Research frontiers

Unfortunately, levels of pancreatic enzymes several days after the DBE procedure and evaluation of potential lesions in the pancreas under normal clinical conditions are unknown in humans. In this study, the authors demonstrated that focal ischemic lesions in the pancreatic parenchyma, and minor enzymatic alterations were related with DBE procedure in a porcine model.

Innovations and breakthroughs

Previous reports have highlighted the importance of the amylase levels in patients before and after oral DBE. Increased levels of amylase and lipase in 51.4% of the patients 24 h after the procedure have been reported. However, only 2.8% of them suffered acute pancreatitis. This is believed to be the first study in an animal model aimed at clarifying the etiology of pancreatic hyperamylasemia and pancreatitis after DBE. Furthermore, the study suggested that a vascular component was probably involved in the etiology of pancreatic alterations after DBE.

Applications

By understanding the etiology of post-DBE pancreatic hyperamylasemia, this study demonstrates the need for further research aimed at evaluating the effects of DBE on the vascular supply to the pancreas.

Terminology

DBE is recognized as the gold standard method for total exploration of the small intestine. Based on the already existent push endoscopy, DBE is a form of deep endoscopy that not only allows the exploration but also treatment of the most common digestive disorders of the small intestine, such as obscure gastrointestinal bleeding, tumors, Crohn's disease and polyps. The equipment consists of an endoscope and an overtube; both of them with a latex balloon attached to the tip. The two balloons are inflated and deflated in an alternating sequence so as to allow the endoscope to progress (pushing phase) or fold the explored intestine behind the balloons (pulling phase).

Peer review

This is an important issue because DBE might be associated with an increase of serum pancreatic enzymes or even complicated by acute pancreatitis. The mechanism of post-DBE pancreatitis has not been fully explained yet. That is why such an experimental study is important to understand possible pathogenetic mechanisms.

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ORIGINAL ARTICLE

Serum levels of microRNAs can specifically predict liver injury of chronic hepatitis B

Hui Zhang, Qing-Ya Li, Zhi-Zhong Guo, Yan Guan, Jia Du, Yi-Yu Lu, Yi-Yang Hu, Ping Liu, Shuang Huang, Shi-Bing Su

Hui Zhang, Qing-Ya Li, Zhi-Zhong Guo, Yan Guan, Jia Du, Yi-Yu Lu, Shi-Bing Su, Research Center for Traditional Chinese Medicine Complexity System, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China
Yi-Yang Hu, Ping Liu, Institute of Liver Disease, Shanghai Shuguang Hospital, Shanghai 201203, China
Shuang Huang, Department of Biochemistry and Molecular Biology, Georgia Health Sciences University, Augusta, GA 30912, United States
Author contributions: Zhang H, Li QY, Guo ZZ, Guan Y and Hu YY collected the samples and did RT-PCR quantification of miRNAs in serum; Zhang H analyzed the data and wrote the first draft of this paper; Huang S and Su SB revised the paper; Zhang H, Liu P and Su SB designed the research; and all authors contributed to the research design, data collection and analysis, and approved the final paper to be published.
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Correspondence to: Shi-Bing Su, PhD, Research Center for Traditional Chinese Medicine Complexity System, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Pudong, Shanghai 201203, China. shibingsu07@163.com
Telephone: +86-21-51323013 Fax: +86-21-51323013
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Abstract

AIM: To investigate whether circulating microRNAs (miRNAs) can serve as molecular markers to predict liver injury resulted from chronic hepatitis B (CHB).

METHODS: The profiles of serum miRNA expression were first generated with serum samples collected from 10 patients with CHB and 10 healthy donors (Ctrls) by microarray analysis. The levels of several miRNAs were further quantitated by real-time reverse transcription polymerase chain reaction with serum samples from another 24 CHB patients and 24 Ctrls. Serum samples of 20 patients with nonalcohlic steatohepatitis (NASH) were also included for comparison. The comparison in the levels of miRNAs between groups (CHB, NASH and Ctrl) was analyzed with Mann-Whitney *U*-test. The correlation between miRNAs and clinical pathoparameters was analyzed using Spearman correlation analysis or canonical correlation analysis. The receiver-operator characteristic (ROC) curves were also generated to determine the specificity and sensitivity of each individual miRNA in distinguishing patients with CHB from Ctrls.

RESULTS: miRNA profile analysis showed that 34 miRNAs were differentially expressed between CHB and Ctrl subjects, in which 12 were up-regulated and 22 down-regulated in CHB subject (fold change > 2.0 and $P < 0.01$). The median levels of miR-122, -572, -575 and -638 were significantly higher ($P < 1.00 \times 10^{-5}$) while miR-744 significantly lower ($P < 1.00 \times 10^{-6}$) in CHB compared with the Ctrl. The levels of miR-122, -572 and -638 were also higher ($P < 1.00 \times 10^{-3}$) while the level of miR-744 lower in CHB ($P < 0.05$) than in NASH, although the difference between them was not as significant as that between CHB and Ctrl. ROC curve analysis revealed that the levels of miR-122, -572, -575, -638 and -744 in serum were sensitive and specific enough to distinguish CHB, NASH and Ctrl. Multivariate analysis further showed that the levels of these miRNAs were correlated with the liver function parameters. Most significantly, it was the scatter plot of principal component with the levels of these miRNAs, but not the parameters of liver function, which clearly distinguished CHB, NASH and Ctrl subjects.

CONCLUSION: Serum levels of miR-122, -572, -575, -638 and -744 are deregulated in patients with CHB or NASH. The levels of these miRNAs may serve as potential biomarkers for liver injury caused by CHB and NASH.

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Key words: Chronic hepatitis B; Nonalcohlic steatohepatitis; Serum microRNAs; Liver injury

Peer reviewers: Dr. Philip Abraham, Professor, Consultant Gastroenterologist, Hepatologist, PD Hinduja National Hospital and Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400016, India; Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor, Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

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INTRODUCTION

Hepatitis B virus (HBV) infection is one of the major health problems in China[1]. Of the 350 million individuals worldwide infected with the HBV, one-third are from China[2]. HBV infection results in chronic hepatitis B (CHB) and patients with CHB exhibit a high risk of developing liver cirrhosis and hepatocellular carcinoma[3]. Although HBV itself is noncytopathic, host immune response often causes liver damage in patients with HBV infection. Currently, the most commonly used markers of liver injury are the enzymatic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in blood; however, these markers are devoid of sufficient sensitivity and specificity to diagnose virus-induced liver damages[4,5]. Therefore, assessing the severity of HBV-induced damages and monitoring the progression of CHB are major clinical challenges.

microRNAs (miRNAs) are evolutionarily conserved, and are small (typically ~22 nt in size) regulatory RNA molecules that modulate the levels of specific targets, and are thus actively involved in a wide range of physiologic and pathologic processes[6,7]. Interestingly, miRNAs are very stable in circulation systems, and tissue or organ-specific intracellular miRNAs can often be detected in blood under pathological conditions[8-12]. The elevated levels of these miRNAs in blood are most likely caused by their release into the circulation system in the processes accompanied with cell death, such as cell turnover, cell destruction and pathological injury[13-16]. For example, the levels of miR-1, a muscle and heart-specific miRNA, is elevated in blood during acute myocardial infarction[13]. miR-141, a miRNA highly expressed in prostate cancer cells, is present at a significantly higher level in prostate cancer patients than healthy donors[14]. In addition, the levels of serum miRNAs can also be associated with different physiological conditions. For instance, miRNAs of presumed placental origin were detected at high levels in the plasma of pregnant women[17]. CHB is an infectious illness, and the host immune response to HBV infection is thus expected to cause both hepatocellular damage and viral clearance[18]. In fact, CHB progresses with significant apoptosis and necrosis of hepatocytes[19]. It is of interest to determine whether particular miRNAs are released to blood of CHB patients and can serve as predictor for CHB liver injury.

The goal of this study is to investigate whether the circulating miRNAs can be used as molecular biomarkers to monitor the pathological development of CHB. As liver cells are also damaged along the progression of nonalcoholic steatohepatitis (NASH) and caused by the build-up of fat cell in the liver[20], we included samples of NASH patients in this study. Through the comparison of serum miRNA expression profiles among CHB and NASH patients and healthy donors, we found that the expression of miR-122, -572, -575, -638 and -744 was deregulated in both CHB and NASH patients. The expression of these five miRNAs was significantly correlated with pathological parameters of liver. We, therefore, conclude that these five miRNAs may serve as potential biomarkers for CHB and NASH-induced liver injury.

MATERIALS AND METHODS

Study subjects and clinical parameters

Sera collected from 34 CHB, 20 NASH patients and 34 healthy donors (Ctrls) were included in this study. Samples from 10 CHB patients and 10 Ctrls were subjected to miRNA microarray analysis to obtain serum miRNA profiles. Those miRNAs with altered levels were further measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) with the samples from the remaining 24 CHB patients and 24 Ctrls. To determine the specificity of miRNA level change, serum samples from 20 NASH patients were also included for qRT-PCR analysis. Serum samples of Ctrls were randomly selected from a collection of 120 individuals who had annual physical examination at Shanghai Shuguang Hospital, Shanghai, China. Samples of CHB and NASH were from patients seeking treatment in Shanghai Shuguang Hospital. The diagnostic criteria for CHB followed the guidelines that defined by the Chinese Society of Hepatology and Chinese Society of Infectious Diseases in 2005[21]. The diagnosis of NASH was based on the guidelines for diagnosis and treatment of nonalcoholic fatty liver diseases that issued by Fatty Liver and Alcoholic Liver Disease Study Group of the Chinese Liver Disease Association in 2006[22]. The clinical parameters of these patients are listed in Table 1. This study was approved by the Institutional Review Board of Shanghai Shuguang Hospital.

Serum sample collection and RNA isolation

All serum samples were derived from freshly-drawn blood and stored at -80 ℃. RNA in serum was isolated using a miRVana PARIS kit (Ambion, Austin, TX, United States) according to the manufacturer's protocol followed by the treatment of RNase-free DNase I (Promega, Madison, WI, United States) to eliminate DNA contamination. The concentration of RNAs extracted from serum ranged from 1.5 to 12 ng/μL.

Table 1 Clinical characteristics of participants in validation

Group	CHB	NASH	Ctrl
Individuals (*n*)	24	20	24
Gender (*n*)			
Male	21	17	19
Female	3	3	5
Age (yr)	37.6 ± 9.0	39.4 ± 9.6	35.6 ± 10.2
ALT (IU/L)	82.6 (14-412)	51.7 (18-203)	21.5 (14-43)
AST (IU/L)	62.6 (9-206)	31.55 (17-62)	21.4 (16-49)
GGT (IU/L)	69.4 (10-580)	45.8 (13-124)	20.8 (12-33)
ALP (IU/L)	95.8 (51-211)	66.0 (41-90)	62.3 (42-96)
TBIL (μmol/L)	19.2 (10.2-50.7)	19.5 (13.1-31.7)	15.9 (6.9-27.1)
HBV DNA	7 377 395 (0-94 800 000)	0	0
Bile acid (μmol/L)	6.9 (1.4-13)	17.9 (3-118.7)	8.4 (4.1-12)
HBV status (*n*)			
$HBsAg^+$	24	0	0
$HBsAg^-$	0	20	24

Bile acid were given as medians (range). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; TBIL: Total bilirubinand; CHB: Chronic hepatitis B; NASH: Nonalcohlic steatohepatitis; Ctrl: Healthy donor; HBsAg: Hepatitis B surface antigen.

Serum miRNA profiling and data analysis

The profiles of serum miRNAs of 10 CHB patients and 10 Ctrls were generated using Agilent Human miRNA microarray V3 (Agilent Technologies Inc, Santa Clara, CA, United States). The microarray chip is comprised of 2371 different probes for a total of 851 human miRNAs. One hundred nanograms of serum RNA was used for each array. The arrays were read using the Agilent microarray scanner and the data were extracted using Feature Extraction V10.7 (Agilent Technologies, CA, United States). All data were transformed to log base 2. The differences between samples were calculated using unsupervised analysis (SAS system, Shanghai Biochip, Shanghai, China). Only the miRNAs with the fold difference > 2.0 and $P < 0.01$ were considered significant.

Validation of internal reference for serum miRNA quantification

There has been no consensus on the reference genes for qRT-PCR analysis of serum miRNAs. However, 6 miRNAs, RNU6B[23], miR-24[14,24], miR-16[15], miR-181a[25], miR-454[26] and miR-638[27], have been reported to be consistently present in human serum. Therefore, these miRNAs were empirically analyzed by qRT-PCR in samples from all patients and Ctrls. The cycle threshold (Ct) values were converted into relative quantities for analysis with geNorm software[28], which selects the optimal number of the most stable genes for normalization. To calculate the expression stability of a given gene (gene stability measure *M*), the program uses an algorithm based on the mean of the pairwise variation of a given reference gene compared to all other control genes. The higher the value of *M* is, the more the expression variability of the corresponding reference gene is. The least stable gene, i.e., the gene with the highest *M* value, was excluded from the subsequent analysis. The remaining genes was recalculated for *M* values and the gene with highest *M* was again excluded until the two most stable genes were left.

Quantification of serum miRNAs

qRT-PCR-based quantification of miRNAs (200 μL of serum from each participant) was performed with Bulge-Loop™ miRNA qPCR Primer Set (Guangzhou Ribobio, Guangzhou, China) and SYBR Green PCR Master Mixture (TOYOBO, LTD, Japan) according to the manufacturer's instructions using a Rotor-Gene 6000 Real-time PCR machine (Corbett Life Science, Sydney, Australia). The specificity of each PCR products was validated by melting curve analysis at the end of PCR cycles. All samples were analyzed in triplicate and the Ct was defined as the number of cycles required for the fluorescent signal to reach the threshold. The levels of miRNAs in serum were calculated using the formula $2^{\Delta Ct}$ where ΔCt = Ct of internal reference -Ct of target miRNA.

Establishment of receiver-operator characteristic curves

Receiver-operator characteristic (ROC) curves were established to evaluate the difference in the levels of serum miRNAs among CHB, NASH and Ctrls. Statistical significance for correlations was calculated using Spearman's non-parametric rank test and the correlation coefficient R generated by Spearman correlation formula.

Statistical analysis

Comparisons between groups were analyzed using Mann-Whitney *U*-test, Pearson χ^2 test, canonical correlation analysis or Spearman correlation analysis wherever appropriate. All tests were two-tailed and $P < 0.05$ was considered statistically significant.

RESULTS

Serum miRNA profiles of CHB patients are distinct from those of Ctrls or NASH patients

To determine whether there was difference in serum miRNA profiles between people with or without CHB, we performed miRNA microarray with RNAs isolated from the sera of 10 CHB patients and 10 Ctrls. Among a total of 851 miRNAs analyzed, 34 of them were differentially expressed between CHB patients and Ctrls (fold change > 2.0 and $P < 0.01$) (Table 2).

In order to validate the serum miRNA profiles generated from microarray, we initially turned our attention to identifying a particular serum miRNA that can be used as an internal control. As the levels of RNU6B, miR-24, -16, -181a, -454 and -638 were previously reported be relatively consistent[14,15,23-26], we measured their levels in 16 serum samples (4 CHB, 4 NASH and 8 Ctrls). We employed GeNorm to calculate the stability values (*M*-values) for these candidate miRNAs and excluded the candidates with the lowest stability (the highest *M* value). The stability value was recalculated until the two most stable miRNAs were predicted. Defining *M*-values below 1.5 as the critical limit, GeNorm data analysis showed that miR-24

Table 2 Differentially expressed miRNAs in chronic hepatitis B patients and healthy donors

miRNA	Fold change	*P* value	CHB (log_2) mean	Ctrls (log_2) mean
hsa-miR-122	8.29	2.99E-03	8.14	5.09
hsa-miR-138	4.23	5.69E-03	2.68	0.60
hsa-miR-638	4.18	2.43E-03	12.62	10.55
hsv1-miR-H1	3.93	7.92E-03	7.67	5.70
hsa-miR-575	3.67	5.69E-03	9.59	7.71
hsa-miR-572	3.36	3.98E-03	7.58	5.83
kshv-miR-K12-3	3.34	2.60E-03	10.19	8.45
hsa-miR-1915	3.12	5.22E-03	11.30	9.66
hsa-miR-623	3.07	4.69E-03	6.85	5.23
hsa-miR-1268	2.81	6.43E-03	9.94	8.45
hsa-miR-939	2.63	3.94E-03	8.81	7.42
hsa-miR-498	2.29	4.30E-03	6.07	4.87
hsa-miR-421	0.37	4.05E-03	0.93	2.38
hsa-miR-598	0.35	5.24E-04	0.67	2.20
hsa-miR-155	0.34	6.40E-03	2.40	3.94
hsa-miR-424	0.33	9.76E-03	3.67	5.26
hsa-miR-23b	0.28	8.42E-03	5.44	7.29
hsa-miR-195	0.27	1.17E-03	1.20	3.10
hsa-miR-487b	0.26	5.46E-03	1.49	3.44
hsa-miR-224	0.25	3.71E-03	1.45	3.45
hsa-miR-495	0.24	2.50E-03	1.16	3.21
hsa-miR-181c	0.22	6.78E-03	1.88	4.03
hsa-miR-654-3p	0.21	8.27E-03	1.99	4.22
hsa-let-7e	0.21	2.52E-03	0.75	2.99
hsa-miR-382	0.21	9.11E-03	1.78	4.02
hsa-miR-17[1]	0.19	9.66E-03	2.47	4.89
hsa-miR-128	0.18	6.17E-03	2.38	4.82
hsa-miR-625	0.18	2.70E-04	2.14	4.61
hsa-miR-30e[1]	0.16	2.94E-03	1.89	4.51
hsa-miR-139-5p	0.16	3.10E-03	2.39	5.03
hsa-miR-30c	0.16	8.92E-03	3.66	6.32
hsa-miR-744	0.15	9.63E-03	2.40	5.10
hsa-miR-374b	0.12	3.05E-03	2.35	5.44
hsa-miR-376c	0.11	4.32E-03	2.91	6.04

[1]MicroRNA (miRNA) cloning studies sometimes identify two about 22 nt sequences miRNAs which originate from the same predicted precursor. When the relative abundancies clearly indicate which is the predominantly expressed miRNA, the mature sequences are assigned names of the form miRNA (the predominant product) and miRNA* (from the opposite arm of the precursor). For example, miR-123 and miR-123* would share a pre-miRNA hairpin, but more miR-123 would be found in the cell. In the past, this distinction was also made with "s" (sense) and "as" (antisense). CHB: Chronic hepatitis B; Ctrl: Healthy donor.

and -181a had the least *M* value (0.656) among these candidate miRNAs, implicating that they were the most stable ones. We thus selected miR-24 as the internal control to standardize differentially presented serum miRNAs in qRT-PCR quantification.

Microarray analysis with 10 CHB and 10 Ctrl samples showed that the median levels of serum miR-122, -572, -575 and -638 were higher while median levels of miR-30c and -744 were lower in CHB patients than those in Ctrls (Table 2). In order to validate these microarray-generated results, RNA was prepared from serum samples of another 24 CHB patients and 24 Ctrls and was subsequently subjected to qRT-PCR to measure the levels of miR-122, -572, -575, -638 and -744. Identical to what we observed with microarray analysis, the levels of miR-122, -572, -575 and -638 were 83.40-, 43.17-, 15.24- and 12.95-fold higher in the sera of CHB patients than those of Ctrls ($P = 1.61 \times 10^{-8}$, $P = 1.20 \times 10^{-6}$, $P = 8.27 \times 10^{-8}$, $P = 2.88 \times 10^{-9}$, respectively) (Figure 1A-D) while the level of miR-744 was 5.11-fold lower in CHB patients than that in Ctrls ($P = 1.04 \times 10^{-7}$) (Figure 1E). The level of miR-30c was a little higher in CHB patients than that in Ctrls although it was not statistically significant (Figure 1F).

To determine how specific these altered serum miRNAs were to CHB, we next examined the levels of these miRNA in serum samples of NASH patients. qRT-PCR showed that the levels of serum miR-122, -638, -575 and -572 were 3.04-, 16.32-, 4.27- and 5.62-fold higher ($P = 6.89 \times 10^{-4}$, $P = 7.50 \times 10^{-5}$, $P = 6.72 \times 10^{-6}$ and $P = 1.14 \times 10^{-7}$, respectively) while the level of miR-744 was 3.75-fold lower in NASH patients than in Ctrls ($P = 2.15 \times 10^{-7}$) (Figure 1A-E). When comparing the levels of these serum miRNAs between CHB and NASH samples, we found that the levels of miR-122, -572 and miR-638 were 27.42-, 2.65- and 3.57-fold higher ($P = 5.83 \times 10^{-7}$, $P = 4.18 \times 10^{-3}$, $P = 8.89 \times 10^{-4}$, respectively) while the level of miR-744 was 1.36-fold lower in CHB patients than in NASH patients ($P = 4.8 \times 10^{-2}$) (Figure 1A-C, E). In contrast, no significant difference was found in the levels of miR-575 and -30c between the serum samples of CHB and NASH patients (Figure 1D and F). These results demonstrated that a subset of miRNAs was differentially present in the sera of CHB patients.

Levels of a subset of serum miRNAs can be used to distinguish CHB patients from NASH patients or Ctrls

To determine whether the levels of serum miRNAs can be used to distinguish patients with CHB from those with NASH or Ctrls, we established ROC curves to analyze the difference in the levels of serum miR-122, -638, -575, -572 and -744 between groups. Comparing CHB subjects with Ctrls, ROC curve areas of miR-122, -638, -575, -572 and -744 were found to be 0.98 (95% CI: 0.88-1.00), 1.00 (95% CI: 0.93-1.00), 0.91 (95% CI: 0.79-0.97), 0.95 (95% CI: 0.85-0.99) and 0.95 (95% CI: 0.84-0.99), respectively. The sensitivity and the specificity of each of these miRNAs were 87.5% and 100%, 100% and 100%, 83.3% and 83.3%, 79.2% and 100%, 91.7% and 95.8%, respectively in the CHB subjects and Ctrls (Figure 2A). These results clearly showed that the levels of serum miR-122, -638, -575, -572 and -744 can distinguish patients with CHB from Ctrls.

We next compared the levels of these serum miRNAs between NASH subjects and Ctrls. ROC curve areas of miR-122, -638, -575, -572 and -744 were 0.80 (95% CI: 0.65-0.91), 0.97 (95% CI: 0.87-0.99), 0.90 (95% CI: 0.77-0.97), 0.85 (95% CI: 0.71-0.94), and 0.96 (95% CI: 0.85-0.99). The sensitivity and the specificity were 95.0% and 62.5%, 95.0% and 95.8%, 90.0% and 79.2%, 100.0% and 66.7%, 100.0% and 95.8%, respectively in NASH subjects and Ctrls (Figure 2B). These results also demonstrated that the levels of these five miRNAs can distinguish patients with NASH from Ctrls. Interestingly, comparison of CHB subjects with NASH subjects implicated that the levels of miR-122, -638, -572 and -744 were use-

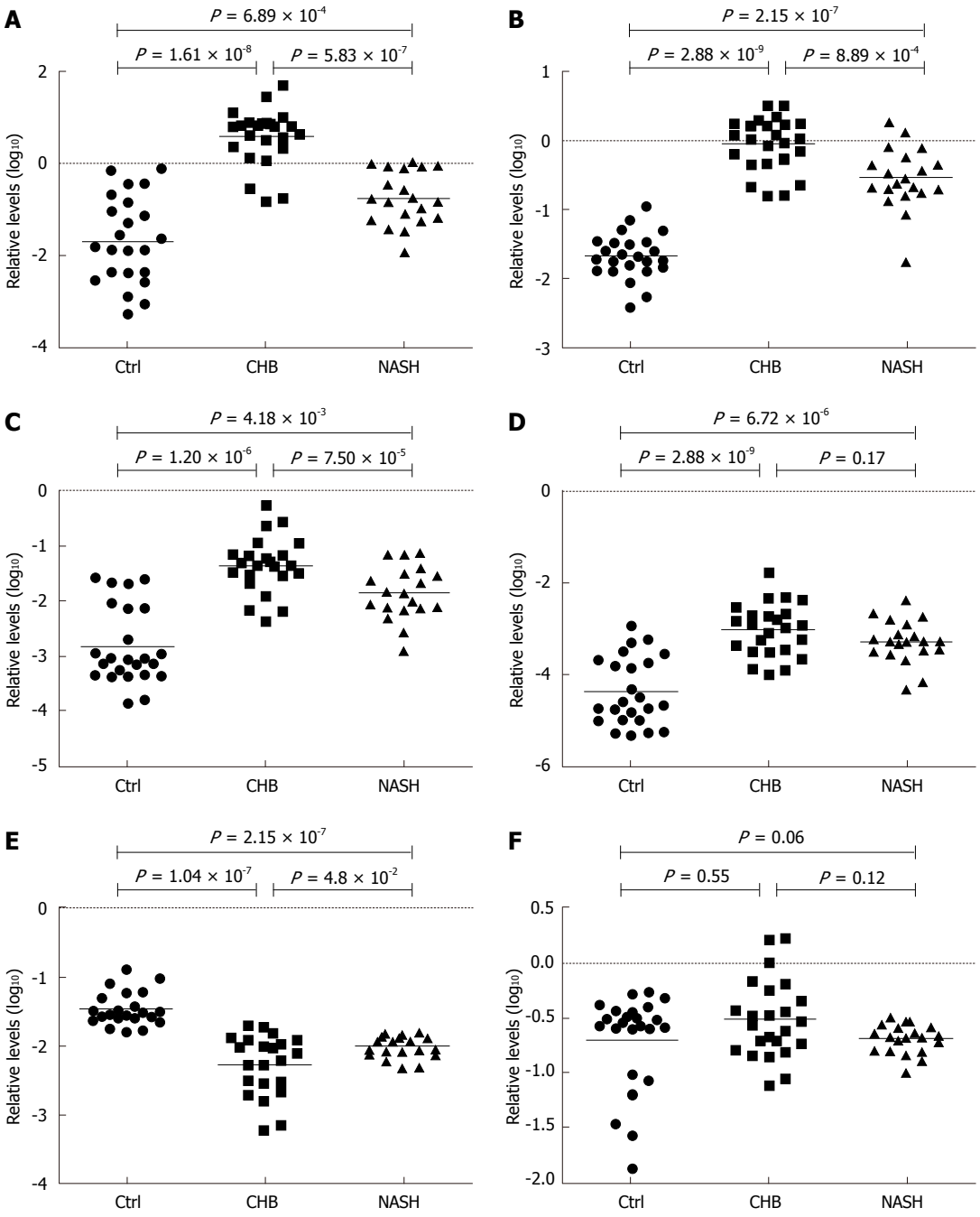


Figure 1 Serum levels of microRNAs in chronic hepatitis B, nonalcohlic steatohepatitis and healthy donors. The levels of serum miR-122 (A), miR-638 (B), miR-572 (C), miR-575 (D), miR-744 (E) and miR-30c (F) in patients with chronic hepatitis B (CHB) (n = 24), with nonalcohlic steatohepatitis (NASH) (n = 20) and healthy donors (Ctrl) (n = 24) were measured by quantitative reverse transcription polymerase chain reaction. The line at each group represents the median value of indicated miRNA. The values are normalized to miR-24 and shown in $\log_{10}$ scale at y-axis. P values on the top are NASH *vs* Ctrl, on the left are CHB *vs* Ctrl and on the right are NASH *vs* Ctrl.

ful markers for discriminating patients with CHB from those with NASH because ROC curve area of miR-122, -638, -572 and -744 were 0.94 (95% CI: 0.83-0.99), 0.79 (95% CI: 0.65-0.90), 0.75 (95% CI: 0.60-0.87) and 0.68 (95% CI: 0.52-0.81) and the sensitivity and the specificity were 87.5% and 100.0%, 83.3% and 70.0%, 75.0% and 75.0%, 50.0% and 85%, respectively, in the two groups (Figure 2C). Together, these results demonstrated that the levels of miR-122, -638, -572 and -744 in serum can be used to distinguish CHB, NASH and Ctrls.

Aberrant levels of serum miR-122, -572, -575, -638 and -744 correlate with the liver pathological parameters

To investigate whether the levels of serum miR-122, -572, -575, -638 and -744 can be used as independent molecular indicators of CHB- or NASH-induced liver injury, we first determined the potential correlation of these five miRNAs in themselves among serum samples from patients with CHB, NASH and Ctrls. Spearman correlation analysis showed that the levels of these five miRNAs in sera were highly correlated among them-

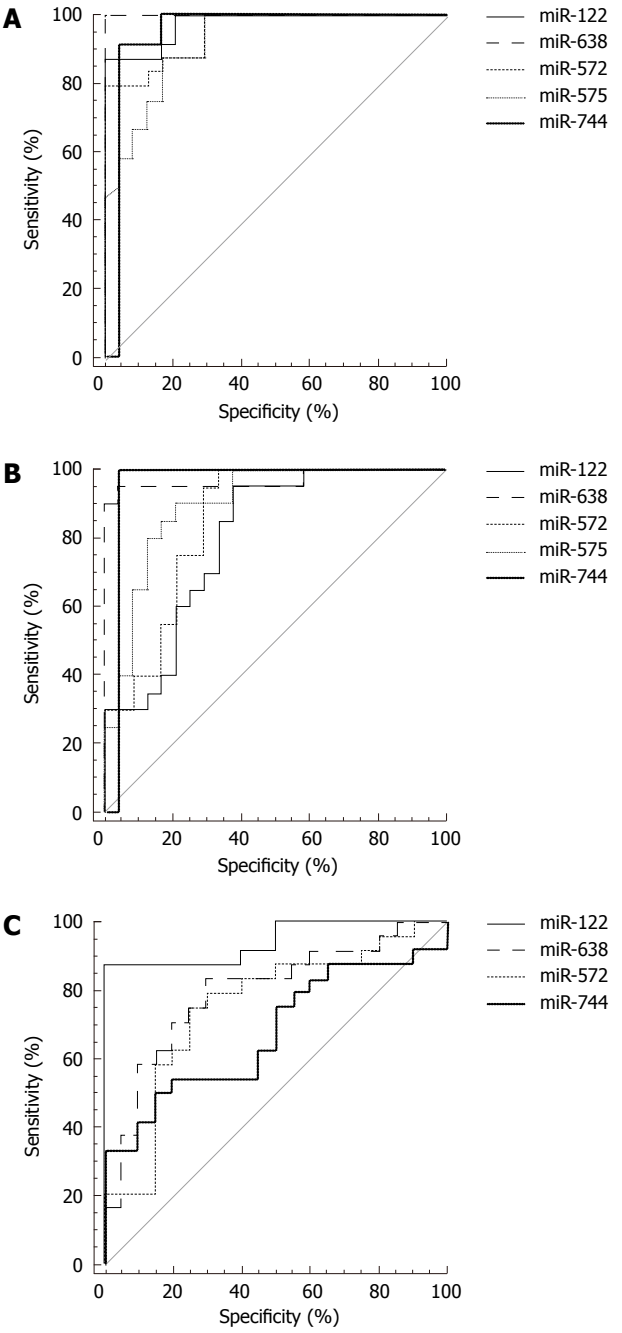


Figure 2 Receiver-operator characteristic curve analyses. Receiver-operator characteristic curves of the miR-122, -638, -572, -575 and -744 were established to discriminate chronic hepatitis B (CHB) from healthy donor (Ctrl) (A), nonalcohlic steatohepatitis (NASH) from Ctrl (B) and CHB from NASH (C).

selves ($r \geqslant 0.57$, $P = 1.00 \times 10^{-4}$; Table 3). We next analyzed the potential correlations between each of these five miRNAs and each of the clinical liver pathological parameters. Spearman correlation analysis showed that correlation only existed between selected miRNAs and selected liver function parameters (Table 3). For example, miR-122 was significantly correlated with both ALT ($r = 0.559$, $P = 1.00 \times 10^{-6}$) and AST ($r = 0.692$, $P = 1.00 \times 10^{-6}$; Table 3). However, none of these miRNAs was correlated with markers of hepatitis viruses including HBsAg, HBeAg and HBV DNA (data not shown).

Table 3 Coefficient of Spearman correlation between microRNA variables and liver function parameter variables (all 68 samples)

Variables	miR-122	miR-638	miR-572	miR-575	miR-744
miR-122	1.000	0.757[b]	0.780[b]	0.614[b]	-0.669[b]
miR-638	0.757[b]	1.000	0.876[b]	0.822[b]	-0.733[b]
miR-572	0.780[b]	0.876[b]	1.000	0.794[b]	-0.639[b]
miR-575	0.614[b]	0.822[b]	0.794[b]	1.000	-0.570[b]
miR-744	-0.669[b]	-0.733[b]	-0.639[b]	-0.570[b]	1.000
ALT	0.559[d]	0.431[d]	0.375[d]	0.299[c]	-0.413[d]
AST	0.692[d]	0.474[d]	0.465[d]	0.324[d]	-0.434[d]
GGT	0.421[d]	0.371[d]	0.280[c]	0.214	-0.355[d]
ALP	0.358[d]	0.312[d]	0.320[d]	0.306[c]	-0.180
TBIL	0.034	0.041	-0.114	-0.074	-0.068
Bile acid	0.068	-0.020	0.008	-0.023	0.090

[b]$P < 0.01$ microRNA (miRNA) *vs* miRNA without superscript b in the same column (2-tailed test); [c]$P < 0.05$ level miRNA *vs* liver function parameter (2-tailed test); [d]$P < 0.01$ level miRNA *vs* liver function parameter (2-tailed test). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; TBIL: Total bilirubin and bile.

In the subsequent studies, we considered both levels of miRNAs and liver pathological parameters as multiple factors and analyzed their correlation using multivariate analysis (canonical correlation analysis). As shown in Figure 3A, the levels of miR-122, -638, -572, -575 and -744 were apparently correlated with liver functional parameters (ALT, AST, γ-glutamyltransferase, ALP, TBIL and bile acid) ($r = 0.74$, $P < 1.00 \times 10^{-4}$). Moreover, the changes in the levels of these miRNAs were greater than those in the values of ALT or AST in the CHB and NASH subjects (Figure 3B). Most significantly, the scatter plot of principal component with the levels of these miRNAs clearly distinguished CHB, NASH and Ctrl subjects (Figure 3C). In contrast, identical analysis with the values of liver functional parameters was unable to distinguish the three groups of subjects (Figure 3D). These data indicated that the profile of miR-122, -572, -575, -638 and -744 in the serum was a better indicator than those well-established liver functional markers for liver injury caused by CHB or NASH.

DISCUSSION

miRNAs can be released into circulating system through damaged cells and tissues. Circulating miRNAs are very stable in plasma and can be found in lipid or lipoprotein complexes[29], apoptotic bodies[30], microvesicles[31] or exosomes[32]. Recent studies have shown that the levels of circulating miRNAs can alter significantly at different physiological stages and pathological conditions. For example, the level of miR-122 (liver specific), miR-133a (muscle specific), and miR-124 (brain specific) are respectively elevated in blood of patients with liver, muscle, and brain injury[9]. Moreover, the level of miR-141 is significantly higher in patients with prostate cancer than in healthy controls[14]. These observations suggest that circulating miRNAs may represent a new class of

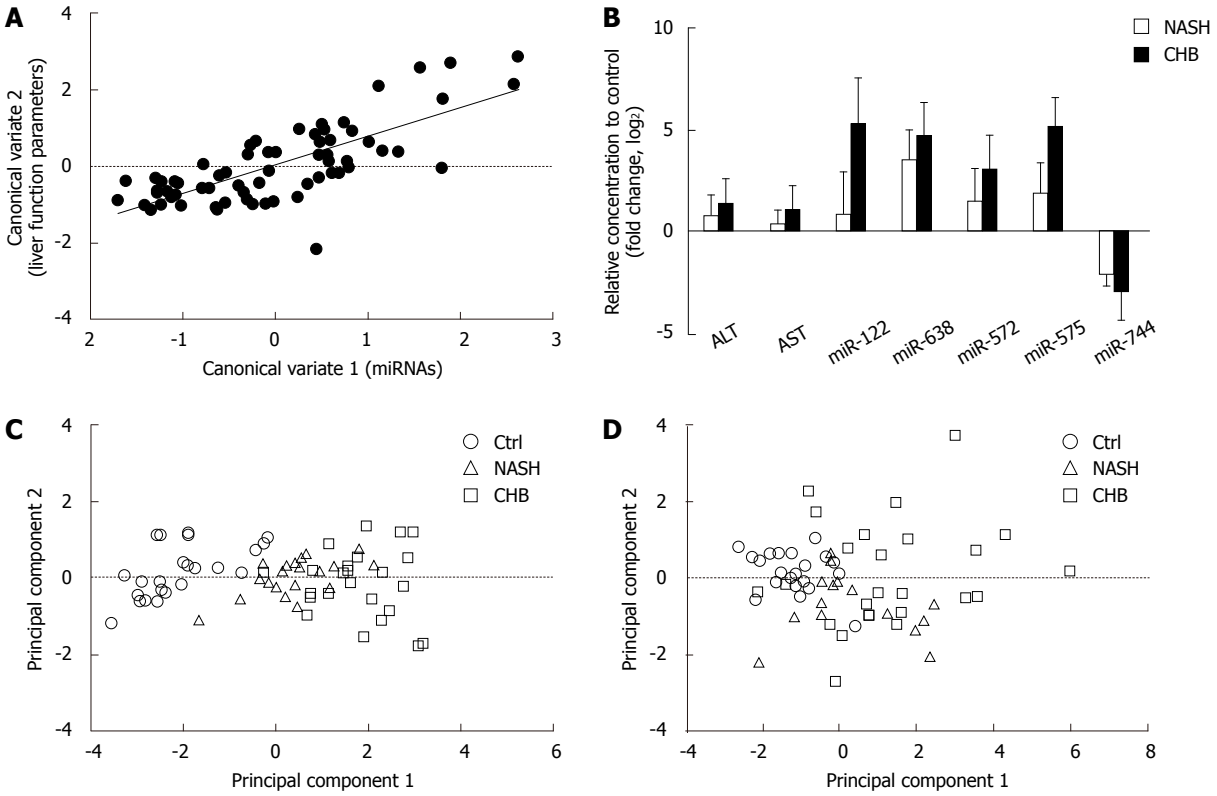


Figure 3 Aberrant levels of serum miR-122, -572, -575, -638 and -744 correlate with the liver pathological parameters. A: Canonical correlation analysis. The correlation between microRNA (miRNA) variables and liver function parameter variables were calculated by canonical correlation analysis. All the data were $\log_{10}$ transformed. Correlation coefficient $r = 0.74$ and $P < 1.00 \times 10^{-4}$. miRNAs: miR-122, -572, -575, -638 and -744; Liver function parameters: Alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), total bilirubin (TBIL) and bile acid; B: Comparison of serum miRNAs, ALT and AST in chronic hepatitis B (CHB) and nonalcohlic steatohepatitis (NASH). The comparison among the levels of ALT, AST, miR-122, -572, -575, -638 and -744 in serum samples collected from patients with NASH and CHB (indicated on x-axis). The relative change of ALT, AST and miRNA expression levels were expressed ratio in $\log_2$ compared with healthy control (Ctrl) (indicated on y-axis). The values of ALT, AST and miRNA fold change are the average of samples from CHB (n = 24), NASH (n = 20) and Ctrls (n = 24), and the SD is shown as an error bar; C, D: Scatter plot of principal components analysis. All the data were $\log_{10}$ transformed to carry out analysis. Scatter plot of first two principal component of miRNAs variables including miR-122, -572, -575, -638 and -744 (C), and liver function parameters including ALT, AST, GGT, ALP, TBIL and bile acid (D) in CHB (n = 24), NASH (n = 20) and Ctrls (n = 24).

biomarkers for monitoring the progress of certain diseases[8-17]. This possibility is supported by several recent reports which demonstrate the potential of miR-9 as a novel non-invasive molecular marker for traumatic spinal cord injury[10], miR-208a for early detection of acute myocardial infarction[11], miR-146a/223 for sepsis[12], miR571 and miR-652 for liver cirrhosis-induced alcoholic hepatitis or hepatitis C[33] and a plasma miRNA panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) for HBV-related HCC[34]. In this study, we further validated that the levels of a subset of miRNAs possess the value as novel and sensitive biomarkers for predicting liver diseases.

miR-122 was previously reported as a liver-specific miRNA[35]. In rodents, liver injury induced by alcohol or chemicals leads to the increased level of plasma miR-122 which occurs earlier than the increase in commonly-used marker ALT[9,36]. Moreover, the level of plasma miR-122 exhibits an excellent correlation with the necroinflammatory activity of HBV[23] and HCV infection[37]. In addition to miR-122, the levels of miR-575, -572, -638 and -744 in serum were also altered in patients with CHB or NASH compared with the Ctrls (Figure 1 and Table 2). Importantly, the alteration of these miRNAs correlated well with well-established liver functional parameters (Figure 3 and Table 3). Our study supports a notion that serum miRNA profile may be used to envisage the occurrence of liver injury caused by CHB and NASH.

CHB and NASH have different histological features in necro-inflammation and fibrosis[18-20]. In this study, the median levels of ALT and AST were significantly higher in CHB than in NASH (ALT: 82.6 U/L *vs* 51.7 U/L, $P < 0.05$; AST: 62.6 U/L *vs* 31.55 U/L, $P < 0.05$) (Table 1). Patients with CHB generally exhibited more severe liver damage than those with NASH, thus explaining the detection of higher levels of serum miR-122, -638 and -572, but lower level of miR-744 in CHB than those in NASH (Figure 1). Current laboratory testing with the established liver function parameters do not reliably predict the type and severity of liver injury[4,5]. It is supported by our observation that scatter plot of principal components analysis with liver function parameters was unable to distinguish CHB from NASH (Figure 3D). However, the changes in the levels of these five miRNAs were significantly greater than those in the values of ALT or AST in the CHB and NASH patients (Figure 3B). Scatter

plot of principal components analysis with the levels of serum miRNAs clearly distinguished CHB, NASH and Ctrls (Figure 3C). In hepatitis, hepatocytes die through the mechanisms of both apoptosis (programmed cell death) and necrosis. Hepatocytes presumably synthesize less AST and ALT in the process of apoptosis[38]. In contrast, either apoptosis or necrosis of hepatocytes will release cellular miRNAs directly into the circulating system. This may explain the reason why the sensitivity of serum miRNAs was superior to ALT or AST in diagnosing liver damages. Although investigations with a larger number of samples may be necessary to fully validate our findings, our results do suggest that the alteration of serum miRNA profile may be a more precise molecular biomarker for predicting the seriousness of liver injury.

This study demonstrated that miR-122, -638, -572 and -575 were presented at higher levels while miR-744 was at lower levels in the sera of patients with CHB and NASH. The levels of these miRNAs were not only correlated with liver pathological parameters, but also were more precise indicators for the type and severity of liver diseases than commonly-used markers such as ALT and AST. In conclusion, analyzing the alteration of serum miR-122, -638, -572, -575 and -744 levels may represent a powerful strategy to diagnose liver injury caused by liver inflammation.

ACKNOWLEDGMENTS

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COMMENTS

Background

microRNAs (miRNAs) are small, non-protein coding transcripts involved in many cellular and physiological mechanisms. Recently, a new class of miRNA called "circulating miRNAs" was found in cell-free body fluids such as serum and urine. Circulating miRNAs have been shown to be very stable, specific, and sensitive biomarkers. In this paper, the authors investigated miRNAs with altered levels in the serum of patients with chronic hepatitis B (CHB).

Research frontiers

Currently, the most commonly used markers of liver injury are the enzymatic activities of alanine aminotransferase and aspartate aminotransferase in blood; however, these markers are devoid of sufficient sensitivity and specificity to diagnose virus-induced liver damages. Therefore, assessing the severity of hepatitis B virus-induced damages and monitoring the progression of CHB are major clinical challenges. Circulating miRNA as a biomarker is a new frontier in diagnostics. The goal of this study was to investigate whether the circulating miRNAs can be used as molecular biomarkers to monitor the pathological development of CHB.

Innovations and breakthroughs

Through comparison of serum miRNA expression profiles among CHB, nonalcohlic steatohepatitis (NASH) patients and healthy donors, the authors found that the expression of miR-122, -572, -575, -638 and -744 was deregulated in both CHB and NASH patients. The authors further showed that the expression of these five miRNAs was significantly correlated with pathological parameters of liver. The authors concluded that these five miRNAs may serve as potential biomarkers for CHB and NASH-caused liver injury.

Applications

The study results suggest that serum levels of miR-122, -572, -575, -638 and -744 are deregulated in patients with CHB or NASH. The levels of these miRNAs may serve as potential biomarkers for liver injury caused by CHB and NASH.

Peer review

The authors compared and analyzed the miRNAs profile of CHB, NASH and healthy control. They found that the level of some miRNAs varied in patients with different clinical diagnosis. For instance, the levels of miR-122, -572, -575, -638 and -744 were significantly correlated with the liver function parameters and they concluded that serum levels of these miRNAs may serve as potential biomarkers for liver injury caused by CHB and NASH. The study is quite interesting and useful for clinical practices, although the results have to be validated by using a larger number of samples.

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ORIGINAL ARTICLE

Inhalation of hydrogen gas reduces liver injury during major hepatotectomy in swine

Lei Xiang, Jing-Wang Tan, Li-Jie Huang, Lin Jia, Ya-Qian Liu, Yu-Qiong Zhao, Kai Wang, Jia-Hong Dong

Lei Xiang, Li-Jie Huang, Lin Jia, Ya-Qian Liu, Yu-Qiong Zhao, Kai Wang, Animal Experiment Centers, PLA General Hospital, Beijing 100853, China
Jing-Wang Tan, Jia-Hong Dong, Institute of Hepatobiliary Surgery, PLA General Hospital, Beijing 100853, China
Author contributions: Dong JH designed the research; Xiang L, Tan JW and Liu YQ performed the research; Jia L, Zhao YQ and Wang K analyzed the data; and Xiang L and Huang LJ wrote the paper.
Correspondence to: Jia-Hong Dong, MD, FACS, Professor, Institute of Hepatobiliary Surgery, PLA General Hospital, Beijing 100853, China. tanjingwang02@yahoo.com.cn
Telephone: +86-10-68937422 Fax: +86-10-68181397
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Abstract

AIM: To study the effect of H_2 gas on liver injury in massive hepatectomy using the Intermittent Pringle maneuver in swine.

METHODS: Male Bama pigs (n = 14) treated with ketamine hydrochloride and Sumianxin Ⅱ as induction drugs followed by inhalation anesthesia with 2% isoflurane, underwent 70% hepatotectomy with loss of bleeding less than 50 mL, and with hepatic pedicle occlusion for 20 min, were divided into two groups: Hydrogen-group (n = 7), the pigs with inhalation of 2% hydrogen by the tracheal intubation during major hepatotectomy; Contrast-group (n = 7), underwent 70% hepatotectomy without inhalation of hydrogen. Hemodynamic changes and plasma concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronic acid (HA), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and malondialdehyde (MDA) in liver tissue were measured at pre-operation, post-hepatotectomy (PH) 1 h and 3 h. The apoptosis and proliferating cell nuclear antigen (PCNA) expression in liver remnant were evaluated at PH 3 h. Then we compared the two groups by these marks to evaluate the effect of the hydrogen in the liver injury during major hepatotectomy with the Pringle Maneuver in the swine.

RESULTS: There were no significant differences in body weight, blood loss and removal liver weight between the two groups. There was no significant difference in changes of portal vein pressure between two groups at pre-operation, PH 30 min, but in hydrogen gas treated-group it slightly decrease and lower than its in Contrast-group at PH 3 h, although there were no significant difference (P = 0.655). ALT and AST in Hydrogen-group was significantly lower comparing to Contrast-group (P = 0.036, P = 0.011, *vs* P = 0.032, P = 0.013) at PH 1 h and 3 h, although the two groups all increased. The MDA level increased between the two group at PH 1 h and 3 h. In the hydrogen gas treated-group, the MDA level was not significantly significant at pre-operation and significantly low at PH 1 h and 3 h comparing to Contrast-group (P = 0.0005, P = 0.0004). In Hydrogen-group, the HA level was also significantly low to Contrast-group (P = 0.0005, P = 0.0005) although the two groups all increased at PH 1 h and 3 h. The expression of cluster of differentiation molecule 31 molecules Hydrogen-group was low to Contrast-group. However, PCNA index (%) was not statistically significant between the two groups (P = 0.802). Microphotometric evaluation of apoptotic index (AI) in terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling-stained tissue after hepatotectomy for 3h, the AI% level in the hydrogen was significantly low to Contrast-group (P = 0.012). There were no significant difference between Hydrogen-group and Contrast-group at pre-operation (P = 0.653, P = 0.423), but after massive hepatotectomy, the TNF-α and IL-6 levels increase, and its in Hydrogen-group was significantly low compared with Contrast-group (P = 0.022, P = 0.013, *vs* P = 0.016, P = 0.012), respectively. Hydrogen-gas inhalation reduce levels of these markers and relieved morphological liver injury and apoptosis.

CONCLUSION: H_2 gas attenuates markedly ischemia and portal hyperperfusion injury in pigs with massive hepatotectomy, possibly by the reduction of inflammation and oxidative stress, maybe a potential agent for treatment in clinic.

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Key words: Massive hepatotectomy; Hydrogen gas; Anti-oxidant; Hyperperfusion; Malondialdehyde; Oxidative stress

Peer reviewers: Ezio Laconi, MD, PhD, Professor of General Pathology, Department of Sciences and Biomedical Technologies, Unit of Experimental Pathology, University of Cagliari, Via Porcell, 4, Ⅳ Piano, 09125 Cagliari, Italy; Dr. Assy Nimer, MD, Assistant Professor, Liver Unit, Ziv Medical Centre, BOX 1008, 13100 Safed, Israel; Takashi Kobayashi, MD, PhD, Department of Surgery, Showa General Hospital, 2-450 Tenjincho, Kodaira, Tokyo 187-8510, Japan

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INTRODUCTION

In hepatotectomy, it is often needed to occlude the portal inflow in order to reducing bleeding, causing liver ischemic injury, the Pringle maneuver, interrupts the blood flow to the liver, produces profound hepatic ischemia and intestinal congestion, it has been used clinically during hepatectomy[1-3]. However, ischemia-reperfusion injury (I/R-I) resulting from the Pringle maneuver is one of the pathogenetic factors involved in postoperative liver dysfunction and hepatic failure, especially when the liver is steatotic and cirrhotic[3-5]. The risk of post-operative liver failure (PLF) or "small of size" syndrome (SFSS) is the central problem in the field of liver resection[3,6]. Oxidative stress is regarded as a major contributor to the development of various hepatic disorders including acute hepatic failure, hepatic fibrosis, and hepatic cancer[7-9]. Protective effect of H_2 gas on liver ischemia reperfusion (I/R) injury and toxic liver injury in rodents has been demonstrated. Previously through ameliorating oxidative stress, H_2 becomes an important potential antioxygen spices agent in clinic[10-12]. However, all experiments about H_2 gas focus on small animal[11,13], and lack of the study in big animal which provide a much more clinically relevant means of investigating the pathophysiology of a disease process. Protective effect of H_2 in big animal can provid more treatment options that can be more readily applied in the human setting. In this study, we investigated firstly the effect of H_2 gas on liver remnant injury in major hepatectomy using the Pringle maneuver in swine, and its feasibility in clinic.

MATERIALS AND METHODS

Animals and husbandry

Fourteen pigs male Bama miniature pigs (15-20 kg) were obtained from the Pig and Poultry Production Institute (Guangxi Zhuang Autonomous Region, China). The swine were raised from a closed herd and kept under strict quarantine protocol. The study was approved by the Hospital Clinic Committee on Ethics in Animal Experimentation. All animals in this study were treated humanely and in accordance with institutional and national guide lines for ethical animal.

Surgical technique

An upper midline incision with right or bilateral subcostal extensions (inverse "L" shape or Mercedesb incision) was performed. The subtotal hepatotectomy with loss of bleeding less than 50 mL, and with hepatic pedicle occlusion for 20 min were performed according to the previous introduce[13]. A 16-gauge catheter was inserted into the main portal vein *via* the gastroduodenal vein to measure the portal vein pressure (PVP).

Study group

Hydrogen-group (n = 7) inhaled with 2% and H_2 98% oxygen supplied through trachea cannula, gas inhalation started once trachea cannula accomplished, ontrast-group (n = 7), only inhale oxygen though tracheal tube. In the two groups, the intraoperative PVP and flow were respectively monitored at the proctectomy, 1 h and 3 h after finishing the hepatectomy.

Serum sample analysis

Blood samples were obtained before laparotomy, posthepatectomy 60 min and 180 min. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronic acid (HA), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) were evaluated. Serum AST, ALT and HA were measured using standard clinical methods for automated analysis (Model 7170; Hitachi Inc, Tokyo, Japan). Plasma TNF-α, IL-6 levels were examined by enzyme-linked immunosorbent assay (ELISA) using a commercial porcine TNF-α/TNFSF2 immunoassay kit (Shanghai Yi Hua Scientifi c, Inc. China). Serum HA levels reflect sinusoidal endothelial damage. HA was measured by a radiometric assay with the Pharmacia HA test (Shanghai Yi Hua Scientific, Inc. China) in prereperfusion and postreperfusion serum samples.

Histological examination

Tissue samples were obtained at 180 min post-hepatotectomy (PH) and were divided into two parts. One was immediately cut into cubes 1 mm and fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 mol/L sodium cacodylate-HCl buffer, pH 7.4) at 4 ℃, prior to sectioning for transmission electron microscopy. Another was fixed with 10% formalin for 24 h and embedded in paraffin. Three-micrometer-thick sections were stained with he-

Table 1 The characteristics of the experiment (mean ± SD)

	Hydrogen-group	Contrast-group	*P* value
BW (kg)	19.2 ± 2.7	18.8 ± 3.1	NS
RLW (g)	302 ± 21	296 ± 23	NS
ELB (mL)	43 ± 12	53 ± 16	NS
OT (h)	2.9 ± 0.3	3.3 ± 0.3	NS

RLW: Removal liver weight; ELB: Estimated loss of bleeding; BW: Body weight; OT: Operating time; NS: Not significant.

matoxylin and eosin and analyzed by the *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method using an apoptosis *in situ* detection kit (Shanghai Yi Hua Scientifi c, Inc. China) according to the manufacturer's instructions[14]. The percentage of TUNEL-positive cells of total cell (apoptosis index, AI) nuclei in 10 high power fields were calculated for the 2 groups then compared. Proliferating cell nuclear antigen (PCNA) is a stable cell cycle nuclear. PCNA protein expression was detected by immunostaining using monoclonal anti-PCN-antibody (Jingmei Biotech Co. Ltd, Shenzhen, China). Data were expressed as the percentage of PCNA-stained hepatocytes per total number of hepatocytes (PCNA index). The mean numbers of PCNA-stained hepatocytes per 10 high power fields were calculated for the 2 groups, divided by the total cell number and then compared.

Malondialdehyde assay in liver tissue

Tissue samples were obtained at pre-hepatotectomy, 60 min, 180 min after hepatectomy for hepatic malondialdehyde (MDA) measurement. Hepatic MDA levels were determined using a agents were purchased from the Nanjing Jiangcheng Bioengineering Institute (Nanjing, China), measured according to the manufacturer's instructions. MDA levels were normalized against protein (pmol/mg).

Serum TNF-α and IL-6 measurement

Serum TNF-α and IL-6 measurement reagents were purchased from the Nanjing Jiangcheng Bioengineering Institute (Nanjing, China). TNF-α and IL-6 ELISA kits (Shanghai Yi Hua Scientifi c Inc., China). TNF-α and IL-6 were measured according to the manufacturer's instructions.

Statistical analysis

Values of parameters are presented as mean ± SD. Statistical significance was determined by Student's *t*-test. Fisher's exact test was used for comparison of adhesions. $P < 0.05$ was considered significant.

RESULTS

Characteristics of the experiment

There were no significant difference between two groups in body weight, removal liver weight, estimated loss of bleeding and operating time (Table 1).

PVP

There was no significant difference in changes of PVP between two groups at pre-operation, PH 30 min (Figure 1). The PVP in hydrogen gas treated-group and moderately increased beyond that measured at laparotomy. The PVP in Contrast-group continue to rise at 3 h of posthepatotectomy, but in hydrogen gas treated-group it slightly decrease and lower than its in Contrast-group, although there no statistical significant differernce ($P = 0.06$).

Hepatocellular injury

The preoperative and serial postoperative measurements of serum ALT and AST, are shown in Figure 2, on which significant differences are noted. There were no significant difference between two groups at pre-operation. After hepatotectomy ALT and AST increased in all of the animals and its in hydrogen gas treated-group was significantly lower comparing to Contrast-group ($P = 0.036$, $P = 0.011$).

Malondialdehyde assay in liver tissue

The serial change of hepatic MDA level in two groups was shown in Figure 3A. Baseline of hepatic MDA between two groups were no significant difference ($P = 0.747$). One hour after massive hepatotectomy, the MDA concentration increased in all the swines. In hydrogen gas treated-group, H_2 gas significantly decreased levels of hepatic MDA, a marker of oxidative stress, the MDA level was significantly low to Contrast-group ($P = 0.0005$, $P = 0.0004$).

Serum HA

The serial change of serum HA level in two groups were showed in Figure 3B. Baseline of HA between two groups were no significant difference ($P = 0.488$). One hour after subtotal hepatotectomy, the HA concentration in serum increased in all the pigs. In Hydrogen-group, the HA level was significantly low to Contrast-group ($P = 0.0051$, $P = 0.0052$).

DISCUSSION

The risk of PLF is the central problem in the field of liver resection[2,3]. This is principally due to the PLF or SFSS, an excessive and destructive portal flow through a remnant liver that is too small, which becomes functionally insufficient[3,4,15], the intraoperative injury including ischemia and inflammatory response is another important pathogenic factors involved in postoperative liver dysfunction and hepatic failure. In studies of extended hepatectomy in dogs, severe damage to the sinusoidal endothelial cells (SECs) of the remnant liver 3 h after the operation was one of the main factors responsible for the high mortality rates[16,17]. Therefore, to reducing the intraoperative injury sometimes is determinant to prevention the PLF or SFSS, when the intraoperative damage is irreversible. It is well known oxidative stress is a major

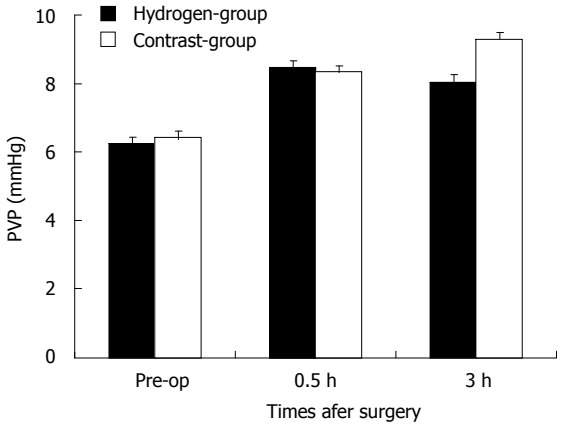


Figure 1 Serial changes of portal vein pressure in two groups. A bar graph shows the mean ± SD of portal vein pressure (PVP) (mmHg) in the two groups. Each group is represented by the mean of 7 swines. There was no significant difference in changes of PVP between two groups at the pre-operation (pre-op), 0.5 h and 3 h.

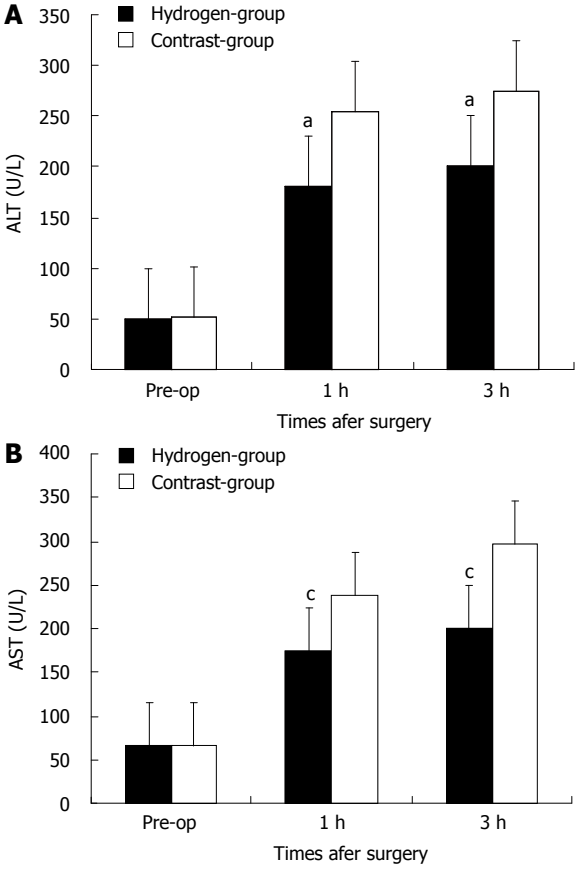


Figure 2 Change of serum alanine aminotransferase level and serum aspartate aminotransferase level in two groups. Each group is represented by the mean of 7 swines. A, B: In hydrogen gas treated-group, the alanine aminotransferase (ALT) (A) and aspartate aminotransferase (AST) (B) levels were significantly lower to Contrast-group. $^{a}P < 0.05$ *vs* ALT level in Contrast-group; $^{c}P < 0.05$ *vs* AST level in Contrast-group. Pre-op: Pre-operation.

contributor to the development of various hepatic disorders including acute hepatic failure, at present, there are no effective agents to alleviate the oxidative stress during clinically operation. However, molecular H_2, has recently been defined as a novel antioxidant, which selectively quenches detrimental the reactive oxygen species (ROS), while maintaining metabolic oxidation reduction reaction and other less potent ROS[10,11,18], indicating it is promising strategies to alleviate intraoperative injury.

In massive hepatotectomy, the intrahepatic vascular space in the remnant liver experiences a drastic reduction, and this leads to portal congestion and hemodynamic instability[4,5,9], the ischemia attenuate the instability, increases the metabolic burden, mitochondria produce more oxygen radicals. During the hepatic inflow occlusion, the intestinal congestion causing the damage of intestine barrier function and the increase of endotoxin absorbtion or bacterial translocation, however, the function of reticuloendothelial system decrease due to the removing of most of liver masss, which contained a lot of phagocyte[6,9,17]. Each individual Kupffer cell in a small-remnant is exposed to higher amounts of endotoxin than that in a whole liver, and triggers Kupffer cells to release a large quantity of free radicals. Lipid peroxidation, which plays a significant role in oxidative damage[18,19], was measured indirectly by assessing the increases in the levels of a lipid peroxidation product, MDA[19-21]. The MDA level is widely used as an indicator of free radical-mediated lipid peroxidation injury. In the present study, serum levels of MDA in contra-group increased rapidly during hepatotectomy (Figure 3A). The observed increase of liver MDA levels was an indicator of lipid peroxidation, which also verified the oxidative damage in the liver tissue in this animal model. While H_2 inhalation inhibited this increase significantly, inhalation of H_2 gas dramatically decreased MDA levels almost to the normal level.

In the Contrast-group and Hydrogen-group, there were significant endothelial denudation in the medium-sized portal vein branches, sinusoidal dilation, hydropic changes of hepatocytes and hemorrhage into perivenular connective tissue, which extended into the hepatic parenchyma (Figure 4B), and there was no intraparenchymal hemorrhage present in H_2 group (Figure 4A). Transmission electron microscopic photographs of the sinusoid was shown (Figure 4C and D). In the Hydrogen-group, the SECs (arrows) and hepatocytes were well preserved, and the structure of the endothelial lining can also be perceived (Figure 4C), in contrast, the sinusoidal endothelial lining was partially destroyed and detached into the sinusoidal space with enlargement of the Disse's spaces (asterisks). Cluster of differentiation molecule 31 (CD31) immunostaining was notable for destruction of the endothelial lining among animals in Contrast-group (Figure 4F) and, in contrast to mild sinusoidal microarchitecture injury in Hydrogen-group (Figure 4E).

Many studies had demonstrated that the high shear-stress or hyperperfusion, due to small liver remnant could cause the sinusoidal endothelial injury, and hepatocyte injury, swelling degeneration of hepatocytes[22,23]. In Contrast-group, the portal overflow damage the 30% liver remnant underwent 20 min ischemia, causing the endothelial denudation, sinusoidal dilation, hydropic changes of hepatocytes and hemorrhage into perivenular con-

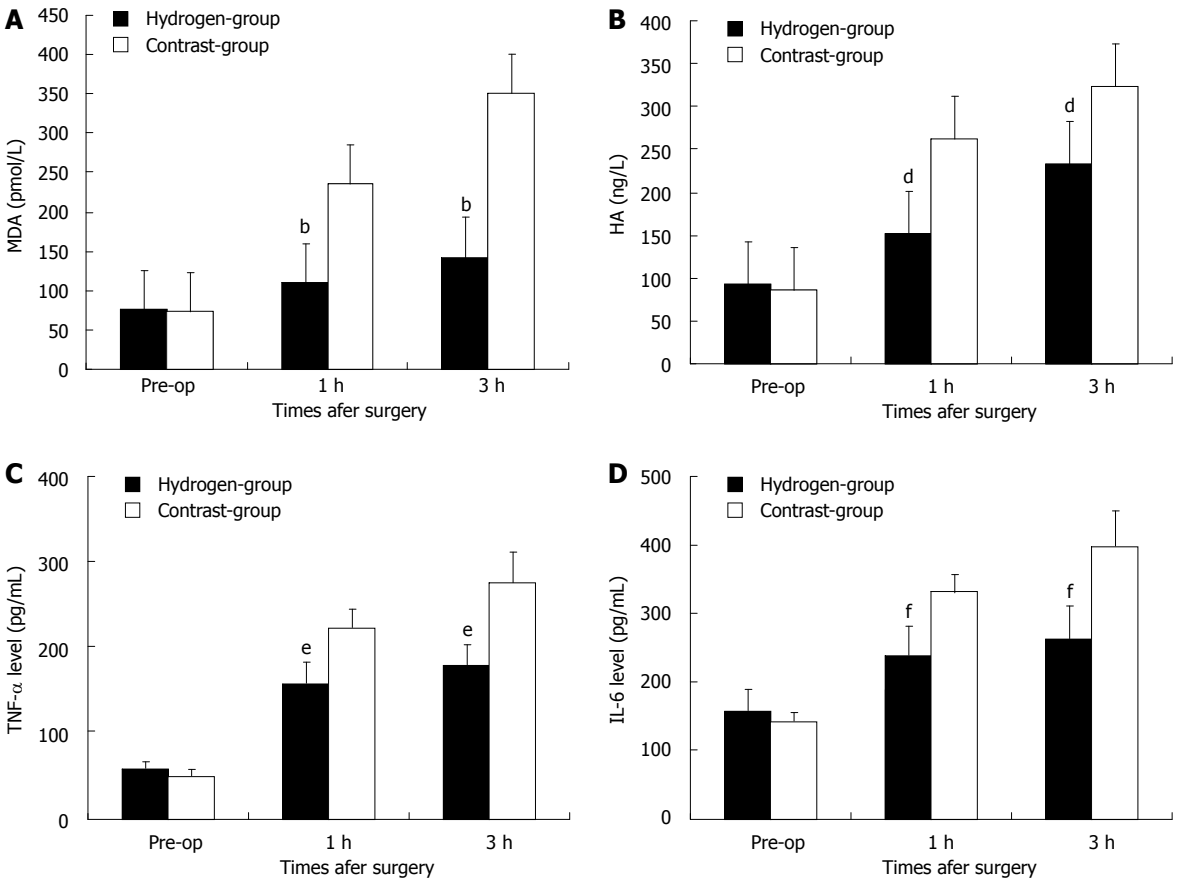


Figure 3 Changes of hepatic malondialdehyde, hyaluronic acid, tumer necrosis factor-α and interleukin-6 levels in two groups. A bar graph shows the mean ± SD of hepatic malondialdehyde (MDA) (A), hyaluronic acid (HA) (B), tumer necrosis factor (TNF)-α (C) and interleukin (IL)-6 (D) level in two groups. Each group is represented by the mean of 7 swines. A, B: In hydrogen gas treated-group, the MDA (A) and HA (B) levels were significantly lower to Contrast-group; C, D: In hydrogen gas treated-group, the TNF-α (C) and IL-6 (D) levels were significantly lower to Contrast-group. Pre-op: Pre-operation. $^{b}P < 0.01$ *vs* MDA level in Contrast-group; $^{d}P < 0.01$ *vs* HA level in Contrast-group; $^{e}P < 0.01$ *vs* TNF-α level in Contrast-group; $^{f}P < 0.01$ *vs* IL-6 level in Contrast-group.

nective tissue, in contrast, the H_2 inhalation alleviated the hyper reperfusion, make the rise in PVP low to Contrast-group, attenuate markedly these injuries (Figure 4A and B). It also identified by the measurement of HA level. HA is synthesized by mesenchymal cells and eliminated in the hepatic sinusoidal endothelium; increased serum HA levels reflect sinusoidal endothelial damage[24,25]. In the present study, an elevation in serum HA level,caused by liver hyperfusion is also significantly low to Contrast-group, indicating the effect of H_2 on hyperfusion injury in the hepatic sinusoidal endothelial (Figure 3B). CD31 immunoglobulin helps maintain endothelial stability by interdigitating with other CD31 molecules at the extracellular border of adjacent cells[26]. The study also showed that hydrogen-inhalation decreased the expression of CD31 molecules (Figure 4E and F), it means the H_2 can reduce the injury of hyperperfusion,and was further demonstrated by the observation of transmission electron microscope examination (Figure 4C and D).

Normal liver has vigorous regenerative potential, portal hyperperfusion is likely to be an important physiologic trigger that stimulates liver regeneration[27,28]. The strength of the regenerative stimulus is proportional to the increase in portal blood flow, as previous shown in experimental animals[29]. The results in the study revealed also there were no significantly difference between the two group at PH 3 h (Figure 5). This means the liver regeneration in the early stage was determined to the portal shear stress, although H_2 decrease free radicals injury. On the other hand, apoptotic cell death is an important contributor to the organ failure common to ALF, even for etiologies thought traditionally to involve mainly hepatocyte necrosis[25,26]. The free radicals may exert a strong cytotoxic effect, and played an important role in inducing apoptosis in the postoperative liver insufficient. The study demonstrated the AI in the hydrogen gas treated-group significantly decrease comparing to the Contrast-group (Figure 6A), this probably attribute to the protection of the H_2 against injury of ROS. Therefore, H_2 play an important role in deceasing the injury of SFSS with decreasing the apoptosis of hepatocyte, without increasing the regeneration.

Even though free radical scavengers have been demonstrated to reduce liver I/R damage[29,30], this is the first observation that the H_2 decreases PVP or hyperperfusion injury in an animal model, which is determinant factor to PLF or SFSS. We observed that H_2 inhalation reduced not only morphological injury, but also serum ALT, AST,

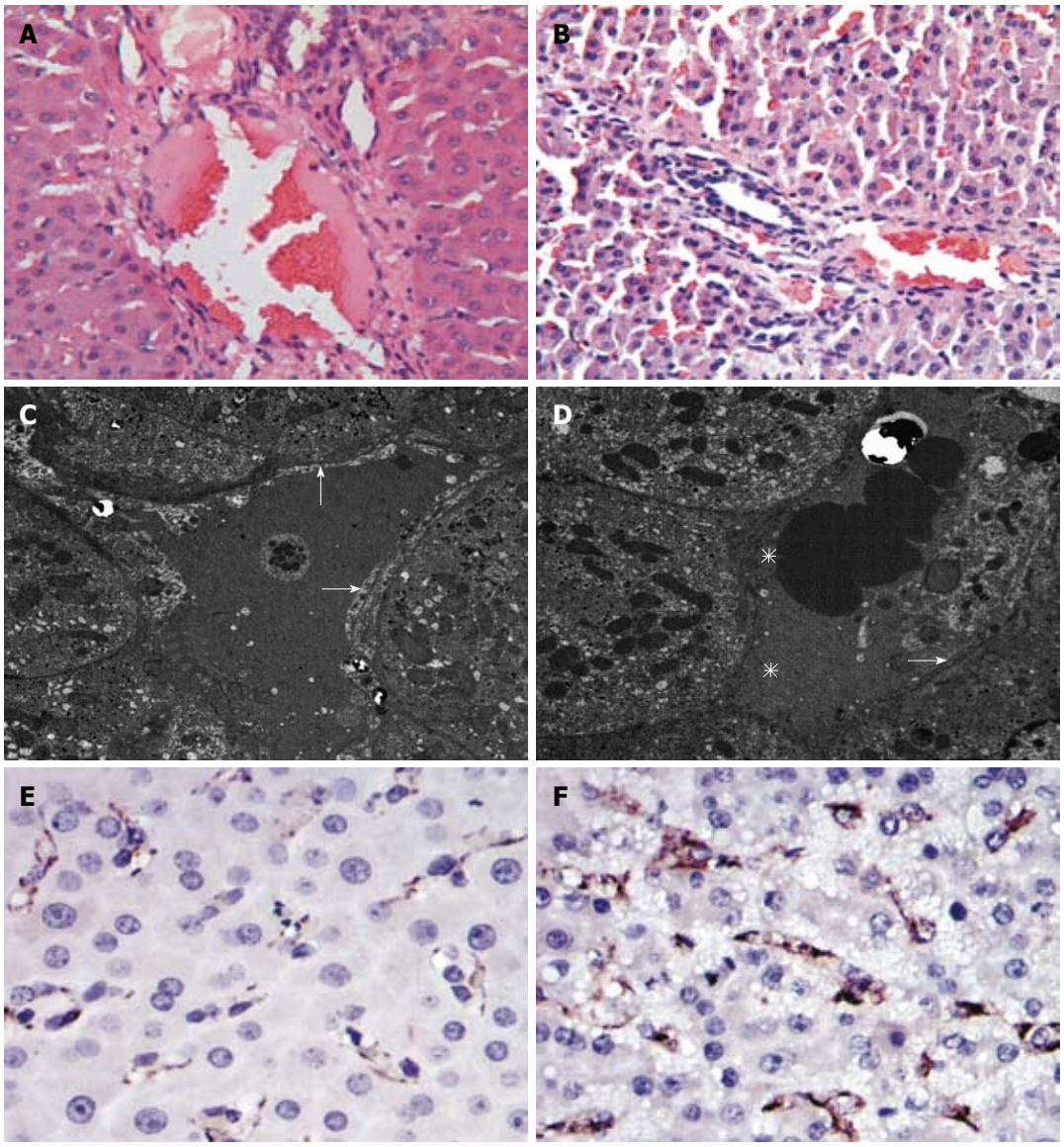


Figure 4 Hematoxylin and eosin, transmission electron microscopic photographs and cluster of differentiation molecule 31 immunohistochemical staining of tissue samples taken 3 h after hepatotectomy. A: Hematoxylin and eosin (HE) staining of the Contrast-group; B: HE staining of the hydrogen gas treated-group; C, D: Transmission electron microscopic photographs of the sinusoid, arrows indicatethe sinusoidal endothelial, asterisks indicate the enlargement of the Disse's spaces; E: Cluster of differentiation molecule 31 (CD31) immunostaining of the hydrogen gas treated-group; F: CD31 immunostaining of the Contrast-group.

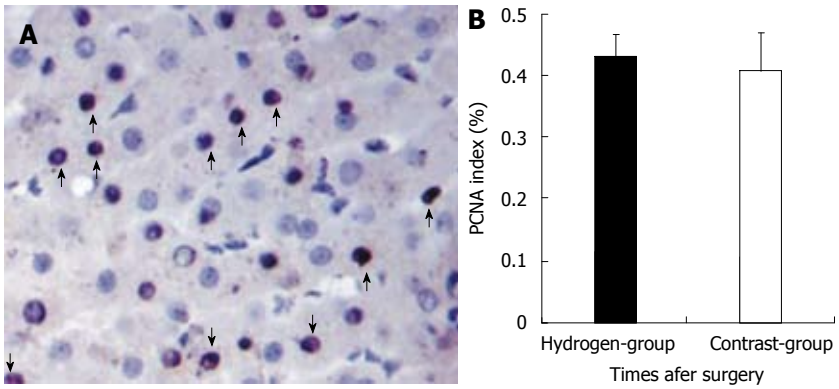


Figure 5 Proliferating cell nuclear antigen immunostaining in liver and the percentage of proliferating cell nuclear antigen stained in two groups. A: Proliferating cell nuclear antigen (PCNA) staining in liver remnant (arrows, positive cell: × 400); B: Microphotometric evaluation in PCNA stained tissue after hepatotectomy for 3 h between two groups. A bar graph shows the mean ± SD of PCNA stained level (%) in two groups. Each group is represented by the mean of 7 swines.

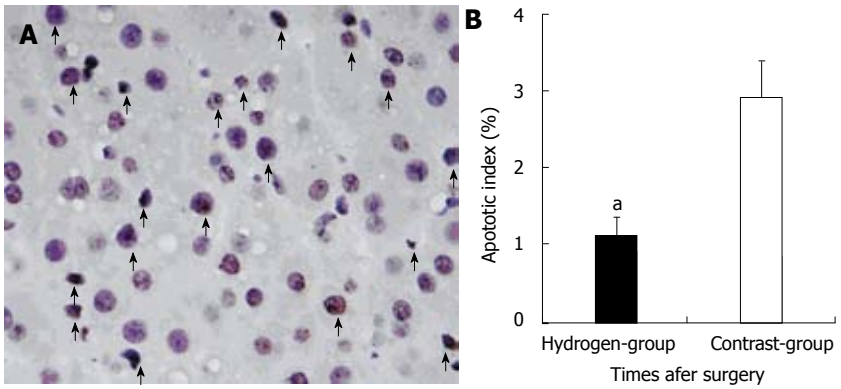


Figure 6 Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining after hepatectomy and protective effect of H_2 against liver apoptotic cell death in two groups. A: Revealed many terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells (arrows, identified morphologically by dark brown staining nuclei) in the liver remnant × 400; B: Microphotometric evaluation of apoptotic index (AI) in TUNEL-stained tissue after hepatotectomy for 3 h. A bar graph shows the mean ± SD of AI level (%) in two groups. Each group is represented by the mean of 7 swines. In hydrogen gas treated-group, the AI level was significantly low to Contrast-group ($^aP < 0.05$ *vs* Contrast-group).

IL-1, TNF-α (Figures 2 and 3C, D). We especially investigated the effect of hydrogen on oxidative stress PVP, and injury/regeneration in this liver hypefusion injury model. The use of gas inhalation to treat diseases has become increasingly popular. There are three endogenous gas include nitric oxide, carbon monoxide and H_2 sulfate. The increased production of these gases under stress conditions may reflect the active involvement of these gases in the protective response[31-33]. However, the inherent toxicity of these gases must be investigated for gas inhalation to be considered an effective therapeutic strategy. H_2 is not produced endogenously in mammalian cells since the hydrogenase activity responsible for the formation of H_2 gas has not been identified.

In conclusion, intraoperative H_2 inhalation in massive hepatotectomy was feasible and can protected the liver injury from hyperperfusion, by reduction of inflammation and oxidative stress, liver remnant apoptosis or necrosis, although it didn't increase the regeneration. However, the exact mechanism and signalling pathway involved in the protection role of H_2 in the small liver remnant injury need to be studied in the future. It is required to fully exploit inhalation of H_2 gas as a therapeutic strategy.

COMMENTS

Background

Hydrogen selectively reduce levels of hydroxyl 1 radicals and alleviates acute oxidative stress in many animal models. But most of these study were used in small animal models and lack of the study in big animal which provide a much more clinically relevant means of investigating the pathophysiology of a disease process. In this study, the authors investigated firstly the effect of H_2 gas on small liver remnant injury or "small of size" syndrome (SFSS) after massive hepatectomy, and its feasibility in clinic.

Research frontiers

It is well known oxidative stress is a major contributor to the development of various hepatic disorders including acute hepatic failure, at present, there are no effective agents to alleviate the oxidative stress during clinically operation. However, molecular H_2, has recently been defined as a novel antioxidant, which selectively quenches detrimental the reactive oxygen species (ROS), while maintaining metabolic oxidation reduction reaction and other less potent ROS, indicating it is promising strategies to alleviate intraoperative injury. Oxidative stress is regarded as a major contributor to the development of various hepatic disorders including acute hepatic failure, hepatic fibrosis, and hepatic cancer. Through ameliorating oxidative stress, H_2 becomes an important potential anti-oxygen spices agent in clinic.

Innovations and breakthroughs

Even though free radical scavengers have been demonstrated to reduce liver ischemia reperfusion damage, this is the first observation that the H_2 decreases portal vein pressure or hyperperfusion injury in an animal model, which is determinant factor to post-operative liver failure or SFSS. It was also firstly demonstrated the feasibility of intraoperative inhalation in big animals. As a kind of gas, intraoperative inhalation was convenient and safe.

Applications

The present study demonstrated firstly the protective effect of H_2 gas on liver ischemia-reperfusion injury (I/R-I), toxic liver injury, and portal hyperperfusion injury in swine, that the physiology is similar to human, indicating intraoperative H_2 gas inhalation will be a treatment modality as potential anti-inflammation response agent in clinic .

Peer review

In this study, many biochemical markers mostly investigated in the study of hepatic I/R-I were evaluated and showed that inhaled hydrogen gas attenuated the I/R-I. This study gives a new insight into the pivotal role of hydrogen gas toward the hepatic I/R-I in clinical settings because hydrogen gas was used in swine model.

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ORIGINAL ARTICLE

Repair of bile duct defect with degradable stent and autologous tissue in a porcine model

Yue-Long Liang, Yi-Chen Yu, Kun Liu, Wei-Jia Wang, Jiang-Bo Ying, Yi-Fan Wang, Xiu-Jun Cai

Yue-Long Liang, Yi-Chen Yu, Kun Liu, Wei-Jia Wang, Jiang-Bo Ying, Yi-Fan Wang, Xiu-Jun Cai, Second Department of General Surgery, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou 310016, Zhejiang Province, China
Yue-Long Liang, Yi-Chen Yu, Yi-Fan Wang, Xiu-Jun Cai, Institute of Minimally Invasive Surgery, Zhejiang University, Hangzhou 310016, Zhejiang Province, China
Author contributions: Cai XJ designed the study; Liang YL, Yu YC, Wang WJ, Liu K, Ying JB carried out the study; Wang YF provided analytic tools and analyzed the data; and Liang YL and Wang YF wrote the paper.
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Correspondence to: Xiu-Jun Cai, MD, Second Department of General Surgery, Sir Run Run Shaw Hospital, Zhejiang University, No. 3, East Qingchun Road, Hangzhou 310016, Zhejiang Province, China. cxjzu@hotmail.com
Telephone: +86-571-86006605 Fax: +86-571-86006605
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Abstract

AIM: To introduce and evaluate a new method to repair bile duct defect with a degradable stent and autologous tissues.

METHODS: Eight Ba-Ma mini-pigs were used in this study. Experimental models with common bile duct (CBD) defect (0.5-1.0 cm segment of CBD resected) were established and then CBD was reconstructed by duct to duct anastomosis with a novel degradable stent made of poly [sebacic acid-co-(1,3-propanediol)-co-(1,2-propanediol)]. In addition, a vascularized greater omentum was placed around the stent and both ends of CBD. Cholangiography *via* gall bladder was performed for each pig at postoperative months 1 and 3 to rule out stent translocation and bile duct stricture. Complete blood count was examined pre- and post-operatively to estimate the inflammatory reaction. Liver enzymes and serum bilirubin were examined pre- and post-operatively to evaluate the liver function. Five pigs were sacrificed at month 3 to evaluate the healing of anastomosis. The other three pigs were raised for one year for long-term observation.

RESULTS: All the animals underwent surgery successfully. There was no intraoperative mortality and no bile leakage during the observation period. The white blood cell counts were only slightly increased on day 14 and month 3 postoperatively compared with that before operation, the difference was not statistically significant (P = 0.652). The plasma level of alanine aminotransferase on day 14 and month 3 postoperatively was also not significantly elevated compared with that before operation (P = 0.810). Nevertheless, the plasma level of γ-glutamyl transferase was increased after operation in both groups (P = 0.004), especially 2 wk after operation. The level of serum total bilirubin after operation was not significantly elevated compared with that before operation (P = 0.227), so did the serum direct bilirubin (P = 0.759). By cholangiography *via* gall bladder, we found that the stent maintained its integrity of shape and was still *in situ* at month 1, and it disappeared completely at month 3. No severe CBD dilation and stricture were observed at both months 1 and 3. No pig died during the 3-mo postoperative observation period. No sign of necrosis, bile duct stricture, bile leakage or abdominal abscess was found at reoperation at month 3 postoperatively. Pigs had neither fragments of stent nor stones formed in the CBD. Collagen deposit was observed in the anastomosis by hematoxylin and eosin (HE) and Masson's trichrome stains. No severe cholestasis was observed in liver parenchyma by HE staining. Intestinal obstruction was found in a pig 4 mo after operation, and no bile leakage, bile duct stricture or biliary obstruction were observed in laparotomy. No sign of bile duct stricture or bile leakage was observed in the other two pigs.

CONCLUSION: The novel method for repairing bile

duct defect yielded a good short-term effect without postoperative bile duct stricture. However, the long-term effect should be further studied.

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Key words: Degradable stent; Bile duct defect; Biliary reconstruction; Autologous tissue; Omentum

Peer reviewers: Atsushi Irisawa, Professor, Department of Gastroenterology, Fukushima Medical University Aizu Medical Center, 10-75, Shiromae, Aizuwakamatsu 960-8555, Japan; Tokihiko Sawada, Professor, Second Department of Surgery, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan; Nobuhiro Ohkohchi, Professor, Department of Surgery, Institute of Clinical Medicine, University of Tsukuba, Tenoudai 1-1-1, Tsukuba 305-8575, Japan

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INTRODUCTION

Laparoscopic cholecystectomy (LC) has been the gold standard procedure for benign gall bladder diseases. Unfortunately, the widespread application of LC has led to a concurrent rise in the incidence of major bile duct injuries (BDI) ranging from 0.3% to 0.65%[1-5]. Occurrence of BDI results in difficult reconstruction, even for experienced hepatobiliary surgeons, and a prolonged hospital stay and a high risk of long-term complications. Roux-en-Y hepaticojejunostomy has been the most commonly used approach for biliary reconstruction, especially in cases of duct transection injury[1,6-9]. But its long-term outcome is still far from satisfied due to the high incidence of reflux cholangitis, choledocholithiasis, anastomotic stenosis caused by scar contracture[10-12] and even canceration[13-15]. In recent years, primary duct-to-duct reconstruction has been used in living-donor liver transplantation and has gained good effects[16-18]. It preserves the function of Oddi's sphincter, which provides a barrier to prevent any reflux into the bile duct[13]. However, major drawbacks, including early ischemic necrosis, leakage, and late anastomotic stricture, cannot be overcome so far. In this research, we created a novel method for bile duct defect repair, and proved its feasibility and safety.

MATERIALS AND METHODS

Animals

Eight experimental Ba-Ma mini-pigs of either sex, weighing 15-20 kg, were provided by Shanghai Multi-Bio-Sci-Tech Co., Ltd., China (license No: SCXK 2005-0002). The animals were housed one per cage at the Experimental Animal Center at Zhejiang University. They were allowed to be accustomed to the laboratory environment for more than one week before the start of the experiment. All animals had free access to water and standard food until the day before surgery. The study was approved by the Ethics Committee of Zhejiang University.

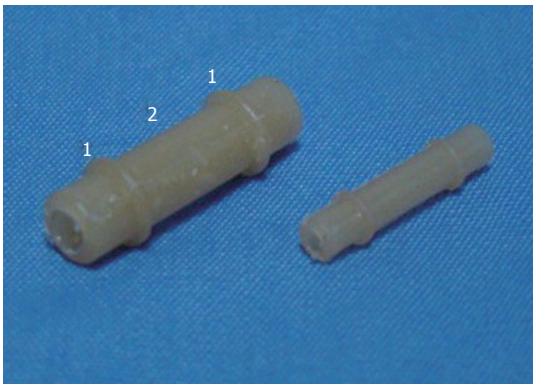


Figure 1 Biodegradable stents of different sizes. 1: Hump rings; 2: Main spindle, 10 mm in length between two hump rings.

Stents

The stent (Figure 1) is made of a novel biodegradable elastomer which is manufactured by the Institute of Polymer Science of Zhejiang University. The elastomer is synthesized with 1,3-propanediol, 1,2-propanediol, and sebacic acid, and then shaped into hollow tube by injecting molding, with a hump ring in both ends. The stent used in the research is 6 mm in external diameter, 1 mm in thickness, and 4 mm in inner diameter. The distance between two hump rings is 10 mm. The stent can completely degrade into carbon dioxide and water in about 3 mo when submerged in fresh human bile *in vitro*. The biocompatible quality of the novel elastomer was approved by the State Food and Drug Administration of China (No. G20090993).

Experimental design

A total of eight Ba-Ma mini-pigs (15-20 kg) were included in this study. The pigs were fasted for 24 h before surgery. Experimental models with common bile duct (CBD) defect (0.5-1.0 cm segment of CBD resected) were established and CBD was reconstructed by duct to duct anastomosis with the novel biodegradable stent. In addition, a vascularized greater omentum was placed around the stent and both ends of CBD. The incidence of jaundice and bile leakage was evaluated. Five pigs were subjected to the examination of complete blood count before operation and on day 14 and month 3 after operation to estimate the inflammatory reaction. Alanine aminotransferase (ALT), γ-glutamyl transferase (γ-GT), serum total bilirubin (TBIL) and direct bilirubin test were also examined pre- and post-operatively to evaluate the liver function. Cholangiography was performed for each pig on months 1 and 3 postoperatively to rule out stent translocation and bile duct stricture. The five pigs were reoperated on to observe anastomosis three months after operation and were sacrificed immediately after that. The

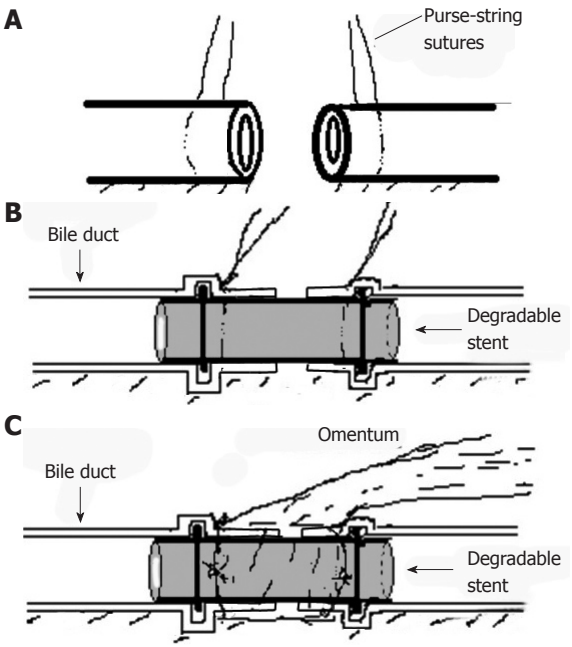


Figure 2 Schematic diagram of repairing bile duct defect with degradable stent and omentum. A: Purse-string sutures with 4-0 Vicryl were made on both ends of bile duct; B: The stent was inserted into both ends of bile duct; C: A vascularized greater omentum was placed around the stent and both ends of common bile duct.

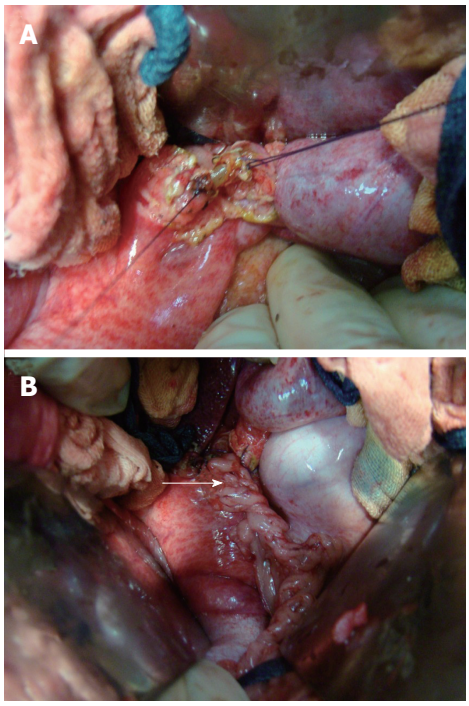


Figure 3 Surgical procedure. A: Stent is placed into common bile duct and two purse-string sutures are tied; B: A layer of large omentum is covered around the stent and two ends of bile duct (arrow).

peritoneal cavity was observed for signs of bile leakage and stricture. The anastomosis was evaluated pathologically, including hematoxylin and eosin (HE) stain and Masson trichrome stain. Liver tissue slides with HE staining were also observed. The other three pigs were raised for one year for long-term observation.

Procedure

Pigs were anesthetized by intramuscular injection of pentobarbital sodium solution (20 mg/mL) with 0.1 mL/kg. Laparotomy was performed *via* a midline incision. Hepatic hilar was dissected firstly to free the CBD. Then a 0.5-1.0 cm segment of CBD under junction of cystic duct was resected. The CBD was reconstructed by duct to duct anastomosis with a stent: purse-string sutures with 4-0 Vicryl (Ethicon, Somerville, NJ, United States) were made on both ends of bile duct (Figure 2A). The stent was inserted into the both ends of bile duct as shown in Figure 2B and 3A. Two ends of bile duct were slightly closed and the purse-string sutures were tied to fix both ends of bile duct on the stent to reconstruct the CBD. A vascularized greater omentum was placed around the stent and both ends of CBD (Figure 2C and 3B).

All pigs were given free access to water, but without food for 24 h postoperatively. They were given half of their normal diet on postoperative day 2. Normal diet was resumed on postoperative day 3.

Statistical analysis

For statistical analysis, the Kruskal-Wallis test was used. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS statistical software package (Version 13.0, SPSS Inc, Chicago, IL).

RESULTS

Surgery was successful in all the cases. There was neither intraoperative death nor bile leakage detected during the observation period. The white blood cell counts were only slightly increased on day 14 and month 3 postoperatively compared with that before operation, the difference was not statistically significant. The plasma level of ALT on day 14 and month 3 postoperatively was also not significantly elevated compared with that before operation. Nevertheless, the plasma level of γ-GT was increased after operation in both groups, especially 2 wk after operation. The plasma level of TBIL after operation was not significantly increased compared with that before operation, so did the plasma level of serum direct bilirubin (Table 1).

By cholangiography *via* gall bladder, we found that the stent maintained its integrity of shape and was still *in situ* on month 1, and it disappeared completely on month 3. No severe CBD dilation and stricture were observed both on months 1 and 3 (Figure 4).

No pig died during the 3-mo postoperative observation period. No sign of necrosis, bile duct stricture, bile leakage or abdominal abscess was found when the animals were reoperated on month 3 postoperatively, and none of the pigs had fragment of stents, and stones formed in the CBD (Figure 5).

Histologically, collagen deposit and bile duct glands proliferation were observed in the anastomosis by hema-

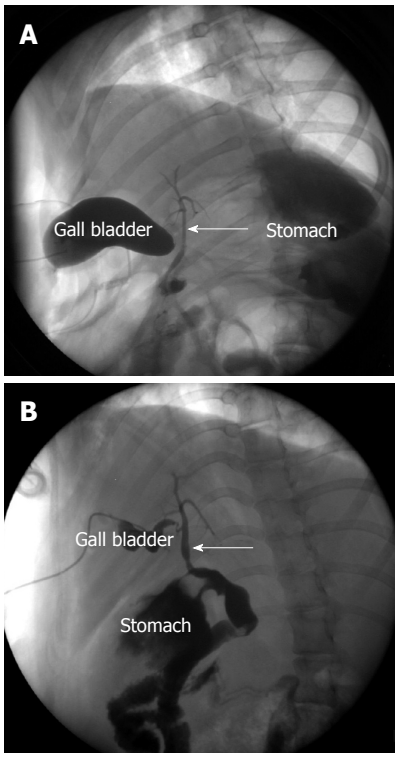


Figure 4 Cholangiography through gall bladder. A: One month after operation, the shape of the stent (arrow) could be visualized in common bile duct; B: Three months after operation, the stent disappeared.

Table 1 Laboratory values

	Before operation	After day 14	Operation month 3	*P* value
WBC	16.62	18.5	18.34	0.652
ALT	51.2	57.2	54.4	0.810
γ-GT	50.2	125.8	72.8	0.004
DBIl	1.1	1.78	1.44	0.227
TBIl	1.76	2.2	2.06	0.759

WBC: White blood cell; ALT: Alanine aminotransferase; γ-GT: γ-glutamic peptidase; DBIl: Direct bilirubin; TBIL: Total bilirubin.

toxylin and eosin and Masson's trichrome stains (Figure 6). No severe cholestasis was found in liver parenchyma by HE staining.

In long-term observation, a pig had intestinal obstruction 4 mo after operation, but no bile leakage, bile duct stricture or biliary obstruction were observed in laparotomy. There was no sign of bile duct stricture or bile leakage in the other two pigs.

DISCUSSION

Bile duct injury is a major complication in biliary surgery such as LC. It can be classified by several classification systems according to the injured site, e.g., Bismuth classification[19] and Strasberg classification[20]. In some cases, bile duct was partially resected and reconstruction with end to end suture was almost impossible for the high incidence of bile leakage and postoperative anastomosis stricture[21,22].

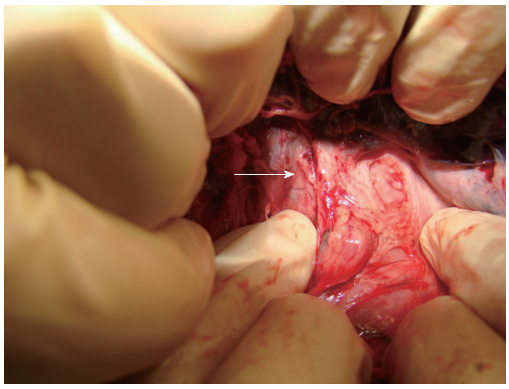


Figure 5 The common bile duct (arrow) three months after operation.

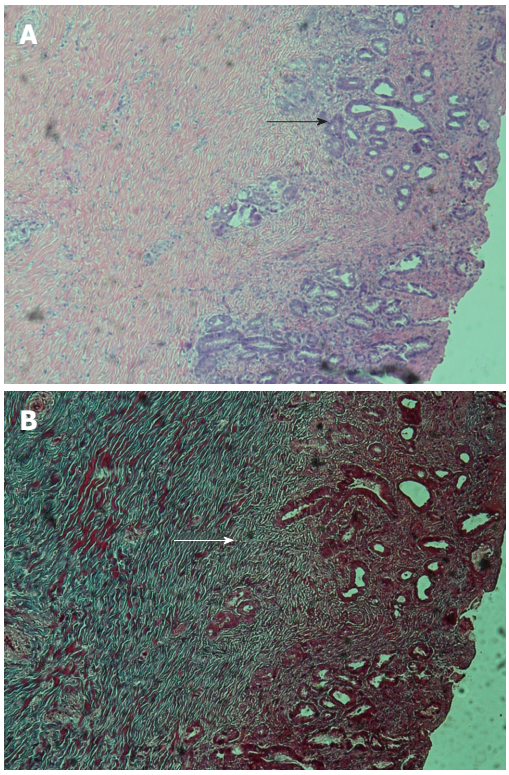


Figure 6 Histology of anastomosis. A: Hematoxylin and eosin stain × 100, black arrow indicates proliferation of bile duct glands; B: Masson's trichrome stain × 100, white arrow indicates deposited collagen fibers.

To keep the biliary continuity and integrality of function is an essential principle for biliary reconstruction. A Roux-en-Y hepaticojejunostomy has been the most commonly used procedure for CBD defect. However, the function of Oddi's sphincter was lost in patients undergoing this procedure, leading to a high incidence of postoperative reflux cholangitis[13,23]. In this research, we aimed to create a new method to repair bile duct defect without sacrificing the function of Oddi's sphincter.

In this procedure, the stent played an important role. The stent was made of degradable material named poly [sebacic acid-co-(1,3-propanediol)-co-(1,2-propanediol)]

(PSPP)[24-26]. Degradable stent had a number of advantages, especially in eliminating the need for stent removal[27,28]. The PSPP belongs to polyester elastomer and possesses a good biocompatibility which is essential for medical applications. The stent connected the two ends of bile duct to maintain the continuity of biliary structure, and it was also used as an inner stent to prevent anastomosis restenoses[22,29,30]. Its degradation time in bile duct is set between 2 and 3 mo, conforming to the healing process of bile duct.

According to the previous reports, the defect of bile duct could be replaced by autologous veins and stents (both silicone stents and biodegradable stents) and they concluded that construction of bile duct appeared to take place by tissue migration[31-34]. Blood supply is important for tissue migration and repair of bile duct defect[35], so vascularized large omentum may be a better autologous tissue compared with veins for temporary repair before bile duct tissue migration was completed. With the assistance of the degradable stent and omentum, we do not need to free much residual bile duct or to mobilize the duodenum to make sure that the anastomosis is tension-free and properly vascularized[36].

Taking into account that bile leakage and bile duct stricture were the two major complications, postoperative cholangiography through gall bladder was repeated to rule out these complications, and liver function was also examined repeatedly to rule out the complication of biliary stricture. The postoperative level of bilirubin and ALT was not significantly elevated compared with that before operation. No obvious bile duct dilatation or stenosis was observed in any pigs by cholangiography. As a result, there was no sign of bile leakage or biliary stricture.

Histologically, collagen deposit and bile duct gland proliferation were observed in the anastomosis three months after operation by HE and Masson's trichrome stains. The neonatal biliary tissue had completely replaced the omentum. Liver parenchyma was also observed by HE stain to rule out cholestasis, and positive result was found. Those results proved that the method is feasible and the biliary tissue can migrate along the surface of stent.

The stent degraded in 3 mo in this research, and the presence of stricture after degradation of the stent is essential to the success of this method, so three pigs were raised for one year to observe its long-term effect. One pig was excluded from long-term observation due to the complication of intestinal obstruction occurring in month 4 after operation and no sign of biliary stricture was found in laparotomy.

This is also a simple method. Only two purse-string sutures are needed without end to end suture and dissection of vein. Vascularized large omentum is easy to mobilize and cover around defect and anastomosis. In addition, two sizes of the stent are available for different situations; the stent would be easily placed into the bile duct if a proper size is selected.

These results suggested that it is a feasible method for repairing bile duct defect, however, the long-term effect (more than one year) should be further observed.

COMMENTS

Background

By the early 1990s, laparoscopic cholecystectomy (LC) had replaced open cholecystectomy as the gold standard procedure for benign gall bladder diseases. Nevertheless, the widespread application of LC has led to a concurrent rise in the incidence of major bile duct injuries. The management of major bile duct injury is a surgical challenge even for experienced hepatobiliary surgeons at tertiary referral centers. Hepaticojejunostomy and duct to duct anastomosis are two commonly used methods for repairing bile duct injury. Despite their advantages, both methods have their critical defects which are difficult to overcome.

Research frontiers

Bile duct injury is a severe complication encountered by almost every hepatobiliary surgeon. The management of major bile duct injury has become a hotspot in clinical studies. With the development of tissue engineering, several researches on biodegradable materials for biliary duct have been conducted.

Innovations and breakthroughs

In this research, the authors created a new method for repairing the bile duct defect with satisfactory effect in a three-month observation period. A novel anastomotic stent and omentum are involved in the new method. By this method, a larger proportion of patients can undergo end-to-end anastomosis and avoid the need for a hepaticojejunostomy. It is also of importance to preserve the function of Oddi's sphincter.

Applications

The study results suggest that repairing bile duct defect by this new method yielded a good short-term effect without postoperative bile duct stricture. It might be applicable in clinical settings in the near future.

Terminology

Elastomer is a class of polymers with a good elasticity. Poly [sebacic acid-co-(1,3-propanediol)-co-(1,2-propanediol)] belongs to polyester elastomer which is considered to have a good biocompatibility.

Peer review

The authors studied the efficacy of the new degradable stent for the bile duct repair in pig experiment. And they concluded that it is a feasible method for repairing bile duct defect. The idea and research of this study are very interesting and applicable for clinical settings in the near future.

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BRIEF ARTICLE

Clinical outcome and predictors of survival after TIPS insertion in patients with liver cirrhosis

Hauke S Heinzow, Philipp Lenz, Michael Köhler, Frank Reinecke, Hansjörg Ullerich, Wolfram Domschke, Dirk Domagk, Tobias Meister

Hauke S Heinzow, Philipp Lenz, Frank Reinecke, Hansjörg Ullerich, Wolfram Domschke, Dirk Domagk, Tobias Meister, Department of Medicine B, University of Muenster, D-48149 Muenster, Germany
Michael Köhler, Institute of Radiology, University of Muenster, D-48149 Muenster, Germany
Tobias Meister, Department of Medicine Ⅱ, Helios Albert-Schweitzer-Hospital, D-37154 Northeim, Germany
Author contributions: Heinzow HS and Lenz P contributed equally to this work, they designed the study, analysed and interpreted the data, and helped with drafting of the manuscript; Lenz P helped with technical and material support; Reinecke F collected the data; Köhler M, Ullerich H, Domagk D showed the transjugular intrahepatic portosystemic stent shunt interventions, and performed critical revision of the manuscript for important intellectual content; Domagk D revised the manuscript for study concept, analysis and interpretation of data; Domschke W revised the manuscript for important intellectual content; Meister T studied the concept, designed, analysed and interpreted data, and helped with drafting of the manuscript, statistical analysis, study supervision and final approval of the version to be published.
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Correspondence to: Tobias Meister, MD, Department of Medicine Ⅱ, Helios Albert-Schweitzer-Klinik, Sturmbäume 8-10, D-37154 Northeim, Germany. tobiasmeister@gmx.de
Telephone: +49-5551-971244 Fax: +49-5551-971420
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Abstract

AIM: To determine the clinical outcome and predictors of survival after transjugular intrahepatic portosystemic stent shunt (TIPS) implantation in cirrhotic patients.

METHODS: Eighty-one patients with liver cirrhosis and consequential portal hypertension had TIPS implantation (bare metal) for either refractory ascites (RA) (*n* = 27) or variceal bleeding (VB) (*n* = 54). Endpoints for the study were: technical success, stent occlusion and stent stenosis, rebleeding, RA and mortality. Clinical records of patients were collected and analysed. Baseline characteristics [e.g., age, sex, CHILD score and the model for end-stage liver disease score (MELD score), underlying disease] were retrieved. The Kaplan-Meier method was employed to calculate survival from the time of TIPS implantation and comparisons were made by log rank test. A multivariate analysis of factors influencing survival was carried out using the Cox proportional hazards regression model. Results were expressed as medians and ranges. Comparisons between groups were performed by using the Mann-Whitney *U*-test and the χ^2 test as appropriate.

RESULTS: No difference could be seen in terms of age, sex, underlying disease or degree of portal pressure gradient (PPG) reduction between the ascites and the bleeding group. The PPG significantly decreased from 23.4 ± 5.3 mmHg (VB) *vs* 22.1 ± 5.5 mmHg (RA) before TIPS to 11.8 ± 4.0 *vs* 11.7 ± 4.2 after TIPS implantation (P = 0.001 within each group). There was a tendency towards more patients with stage CHILD A in the bleeding group compared to the ascites group (24 *vs* 6, P = 0.052). The median survival for the ascites group was 29 mo compared to > 60 mo for the bleeding group (P = 0.009). The number of radiological controls for stent patency was 6.3 for bleeders and 3.8 for ascites patients (P = 0.029). Kaplan-Meier calculation indicated that stent occlusion at first control (P = 0.027), ascites prior to TIPS implantation (P = 0.009), CHILD stage (P = 0.013), MELD score (P = 0.001) and those patients not having undergone liver transplantation (P = 0.024) were significant predictors of survival. In the Cox regression model, stent occlusion (P = 0.022), RA (P = 0.043), CHILD stage (P = 0.015) and MELD score (P = 0.004) turned out to be independent prognostic factors of survival. The anticoagulation management (P = 0.097), the porto-systemic pressure gradient (P

= 0.460) and rebleeding episodes (P = 0.765) had no significant effect on the overall survival.

CONCLUSION: RA, stent occlusion, initial CHILD stage and MELD score are independent predictors of survival in patients with TIPS, speaking for a close follow-up in these circumstances.

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Key words: Transjugular intrahepatic portosystemic stent shunt; Liver cirrhosis; Ascites; Gastrointestinal hemorrhage; Treatment outcome

Peer reviewers: Giulio Marchesini, Professor, Department of Internal Medicine and Gastroenterology, Alma Mater Studiorum University of Bologna, Policlinico S Orsola, Via Massarenti 9, 40138 Bologna, Italy; Roberto Testa, Professor, Department of Internal Medicine, University of Genoa, Viale Benedetto XV 6, 16132 Genoa, Italy

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INTRODUCTION

Portal hypertension is a common problem in gastroenterology and the treatment of its complications is still a challenging task. Major complications of liver cirrhosis and portal hypertension include variceal bleeding (VB) and refractory ascites (RA)[1]. Despite a wide range of therapeutic modalities, including medical and surgical treatments, there is ongoing debate about the most effective treatment algorithm for the complications of portal hypertension[2-5].

At the end of the 1980's a new nonsurgical procedure was developed to enable decompression of the portal circulation *via* expandable metal stents between hepatic veins and the intrahepatic portal vein system - the transjugular intrahepatic portosystemic shunt (TIPS)[6-8]. Since then, the method has been established and improved systematically, culminating in the actual guidelines of the American Association for the Study of Liver Diseases (AASLD)[9].

Most clinicians agree that TIPS has an excellent hemostatic effect in VB (95%), with low rebleeding rates (< 20%)[10]. When endoscopic hemostasis of esophageal varices fails, TIPS becomes the first-line treatment of choice, with an estimated technical success rate in the range of 93%-100%[11-13]. Due to the circulatory effects on portal hypertension, TIPS is also an interesting approach in cases of RA[14-18] and hepatorenal syndrome[19]. However, following TIPS higher rates of hepatic encephalopathy are observed in patients with cirrhosis and RA[11]. Additionally, TIPS insertion has been reported to be successful in patients with portal vein thrombosis[20,21], Budd-Chiari syndrome[22] and portal cavernoma[23].

The use of bare metal stents has been the gold standard in TIPS procedure[24], but the higher occlusion rate with consecutive bleeding complications has recently led to the development of covered metal stents with significantly lower occlusion rates after TIPS implantation[25-28].

In a retrospective single centre study, we evaluated the efficacy and safety of TIPS in the treatment of portal hypertension using a self-expanding bare metal mesh-wire stent. The major objectives of the present study were to observe stenosis and occlusion rates, occurrence of rebleeding and predictors of survival.

MATERIALS AND METHODS

Patients

This retrospective single center study was conducted at the tertiary referral center of Muenster University Hospital (Department of Medicine B). One hundred and one patients were initially scheduled for TIPS implantation. Eventually 81 patients with complications of portal hypertension were enrolled from 1998 until 2008. Twenty patients were excluded because TIPS insertion was technically not feasible. The indication for TIPS treatment included acute or recurrent VB and RA.

Objectives of the study

Endpoints for the study analysis were: technical success (completed TIPS insertion, lowering of the portosystemic pressure gradient), rates of stent occlusion and stent stenosis, rebleeding, RA and mortality. Clinical records of patients were collected and carefully analysed. Baseline characteristics (e.g., age, sex) were retrieved as shown in Table 1.

Definitions

According to Bureau *et al*[29], the following definitions were used:

Stent dysfunction: > 50% reduction of the lumen of the stent at angiography with an increase of the portosystemic pressure gradient of more than 50% of the initial post-interventional value.

Recurrent VB: Recurrent VB that did not respond to the usual pharmacological and endoscopic therapy[30].

RA: Ascites that did not respond to conservative (low-salt diet) and pharmacological (diuretics) treatment or lack of treatment options because of treatment-induced complications[31].

Transjugular intrahepatic portosystemic stent procedure

All TIPS procedures were conducted in strong collaboration with an interventional radiologist and gastroenterologist at our hospital using standard techniques[32]. Through a transjugular venous approach, the right hepatic vein was catheterized. An intrahepatic branch of the portal vein was punctured. Before dilation of the liver parenchyma

Table 1 Baseline characteristics

Variables	Bleeding	Ascites	*P* value
Patients	54	27	
Age (yr)			0.497
mean ± SD	61.7 ± 10.4	63.3 ± 10.8	
Range	38-79	46-84	
Sex (male/female)	33/21	15/12	0.634
CHILD score			
A	24	6	0.052
B	28	18	NS
C	2	3	NS
MELD score	9.4 ± 4.9	13.7 ± 5.2	< 0.001
Underlying disease			
Chronic viral hepatitis B/C	7/1	2/1	0.949
Alcohol abuse	38	21	NS
Autoimmune hepatitis	2	0	NC
PSC/PBC	3	0	NC
Cryptogen	3	3	NS
Re-bleeding after TIPS	13	0	NC
PPG before TIPS (mmHg)	23.4 ± 5.3	22.1 ± 5.5	0.765
PPG after TIPS (mmHg)	11.8 ± 4.0	11.7 ± 4.2	0.883
Stent diameter[1] (mm)			
< 12/≥ 12	6/44	4/23	0.728
Anticoagulation after TIPS	31	9	0.042
LTX after TIPS	7	1	0.131
Median survival time (mo)	> 60	29	0.009
Number of radiological controls until evaluation	6.3 ± 4.8	3.8 ± 3.1	0.029
Time interval until first radiological control	9.3 ± 10.6	4.5 ± 5.6	0.133

[1]In four patients data acquisition of stent diameter not available. NS: Not significant; NC: Not calculated; LTX: Liver transplantation; TIPS: Transjugular intrahepatic portosystemic stent; PPG: Portal pressure gradient; MELD: Model for end-stage liver disease; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis.

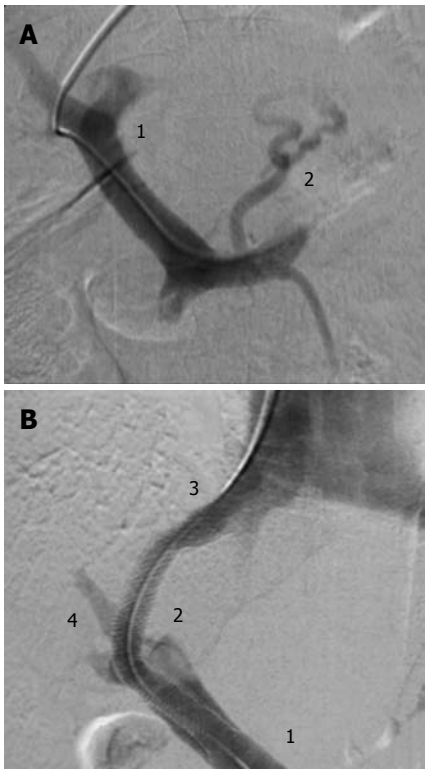


Figure 1 Fluoroscopic images showing transjugular intrahepatic portosystemic shunt placement procedure. A: Portogram after catheterisation of the portal vein, showing perfusion of the portal vein system (1) and oesophageal varices (2); B: Portogram after transjugular intrahepatic portosystemic stent placement. Contrast can be seen in the portal vein (1), through the shunt (2) flowing into the hepatic vein and inferior vena cava (3). Decompression of the portosystemic pressure can be seen in reduced contrast in the portal branch (4). The varices can no longer be identified in the fluoroscopic image.

both the portal pressure and the blood pressure of the right atrium were measured. Then the optimal stent length was defined using a special catheter with opaque markers. After the deployment of the bare metal stent the pressures of the portal vein and the right atrium were measured again. Pressures were measured using an Exadyn transducer set (Braun, Melsungen, Germany). The portal pressure gradient (PPG) resulted as the difference of the portal pressure minus the right atrium pressure (Figure 1). Postinterventional Doppler ultrasonography was carried out the day after TIPS insertion assessing stent patency. As presented by Sahagun *et al*[33] in 1997 shunt stenosis of bare metal stents can effectively be treated by interventional techniques to maintain patency. Stent stenosis due to endothelial growth usually occurs after 3 mo. It was therefore the policy of our institution to reevaluate each patient regularly with Doppler ultrasonography every 3 mo. Interventional angiography was performed every 12 mo or earlier when there was sonographic evidence of stenosis (fall of the initial increase of the portal blood velocity after stenting by > 50% according to Biecker *et al*[34]) or clinical features of recurrent portal hypertension (e.g., hepatic encephalopathy, worsening ascites, presence of high-risk varices at endoscopy or re-bleeding). A TIPS reintervention was performed, when a restenosis or occlusion was affirmed during the angiographic follow-up examination.

Model for end-stage liver disease score

To judge the clinical status of each cirrhotic patient, the model for end-stage liver disease score (MELD score) was calculated based on creatinine, bilirubin and clotting time.

The MELD score for each patient was computed according to the modified method of Wiesner *et al*[35]. This approach differs from the method originally published method by Malinchoc *et al*[36] in two ways: firstly, to avoid negative scores, laboratory serum creatinine levels that were less than 1 mg/dL were rounded off to 1. Preliminary studies in cohorts of non-transplantation candidates have implied that inclusion of the liver disease diagnosis variable does not increase the predictive value of the MELD score; secondly, as previously described by Wiesner *et al*[37,38], 6.43 points as a constant for liver disease aetiology was added to each patient's score to make the results comparable to the originally published studies. The following MELD equation was applied to calculate the severity score: 3.78 [Ln serum bilirubin (mg/dL)] + 11.20 (Ln international normalized ratio) + 9.57 [Ln serum creatinine (mg/dL)] + 6.43.

Table 2 Median survival times depending on various parameters

Parameter	Survival (mo)	95% CI (mo)	Tests	*P* value
Stent open	> 60	NC	Stent occluded *vs* open	0.027
Stent occluded at first control	50	36.6-63.4		
Ascites prior to TIPS	29	1.36-56.64	Ascites *vs* bleeding	0.009
Bleeding prior to TIPS	> 60	NC		
LTX after TIPS	> 60	NC	LTX *vs* no LTX	0.024
Stent diameter < 12 mm or ⩾ 12 mm	> 60	NC	Stent diameter < 12 mm *vs* ⩾ 12 mm	0.486
Anticoagulation	50	14.8–85.2	Anticoag *vs* no anticoag	0.060
No anticoagulation	> 60	NC		
PPG < 12 mmHg or ⩾ 12 mmHg after TIPS	> 60	NC	PPG < 12 mmHg *vs* ⩾ 12 mmHg	0.507
Age (yr)				
⩾ 65	51	33.4-68.6	Age < 65 yr *vs* ⩾ 65 yr	0.053
< 65	> 60	NC		
CHILD score				
A	48.9	40.6-57.2	CHILD A *vs* B	0.013
B	40.0	32.7-47.4		
C	15.0	1.4-28.6		
MELD score				
⩽ 10	52.2	46.3-58.0	MELD score ⩽ 10 *vs* > 10	0.001
> 10	35.3	26.9-43.6		

LTX: Liver transplantation; TIPS: Transjugular intrahepatic portosystemic stent; PPG: Portal pressure gradient; MELD: Model for end-stage liver disease; 95% CI: 95% confidence interval; NC: Not calculable.

Statistical analysis

Data were analyzed using SPSS 17.0 (Chicago, IL, United States). Results are expressed as medians and ranges. Comparisons between groups were performed by using the Mann-Whitney *U*-test and the χ^2 test as appropriate. $P < 0.05$ was considered statistically significant.

For screening of risk factors, univariate analysis was performed. The Kaplan-Meier method was employed to calculate survival from the time of TIPS implantation and comparisons were made by log rank test. A multivariate analysis of factors influencing survival was carried out using the Cox proportional hazards regression model.

RESULTS

Patient characteristics

In the study period (1998-2008), a total of 81 patients were admitted to the study with a mean age of 62.2 ± 10.5 years (range: 38-84 years). According to the indication for TIPS implantation, the patient cohort was subdivided into two groups: VB (group A) and RA (group B). The baseline characteristics of the study population are given in Table 1.

The aetiology of cirrhosis was related to chronic viral hepatitis B or C, alcohol abuse, autoimmune hepatitis and primary sclerosing cholangitis/primary biliary cirrhosis. The mean age in the VB and the RA group showed no statistical difference (61.7 years *vs* 63.3 years, $P = 0.497$). Likewise, the male/female ratio in both groups was comparable, with a slight trend to male patients. The severity of liver disease was calculated according to the CHILD scoring system[39]. Overall, 37% of patients with CHILD A, 57% with B and 6% with CHILD C were enrolled in this study. The MELD score in the RA group was significantly higher compared to the VB group (13.7 ± 5.2 *vs* 9.4 ± 4.9, $P = 0.001$).

TIPS shunt function and patient survival

The PPG significantly decreased from 23.4 ± 5.3 mmHg (VB) *vs* 22.1 ± 5.5 mmHg (RA) before TIPS to 11.8 ± 4.0 mmHg *vs* 11.7 ± 4.2 mmHg after TIPS implantation ($P = 0.001$ within each group). On the other hand, gradient reduction in the VB group did not statistically differ from that in the RA group. Referring to stent diameters there were no relevant differences between both groups. Anticoagulation therapy with enoxaparin at weight-calculated dose was applied for 12 wk after TIPS implantation in 50% of the patients. Thirty-one out of 54 patients in the bleeding group received subcutaneous anticoagulation therapy after TIPS, while only 9 out of 27 patients with RA were anticoagulated post-procedurally ($P = 0.042$). Neither the stent occlusion rate nor the rebleeding rate depended on the anticoagulation state ($P = 0.7$ and $P = 0.47$, respectively). In our patient cohort, the median patency rate of the TIPS shunt was 10 mo. The median survival time was > 60 mo in the VB group *vs* 29 mo in the RA group, showing a significant difference ($P = 0.009$). The number of radiological controls for stent patency was 6.3 ± 4.8 (VB) *vs* 3.8 ± 3.1 (RA) ($P = 0.029$). The mean time interval until the first radiological control was 9.3 ± 10.6 mo (VB) *vs* 4.5 ± 5.6 mo (RA) ($P = 0.133$).

Kaplan-Meier calculation indicated that the stent function (open *vs* occluded) at first control was a significant predictor of survival ($P = 0.027$) (Table 2 and Figure 2B). Furthermore, the median survival time was longer in patients with TIPS due to VB compared to that in patients with RA ($P = 0.009$) (Table 2 and Figure 2A). Seven patients in the VB group and one patient in the RA group underwent liver transplantation. As expected, in univariate analysis survival rates were significantly higher after liver transplantation ($P = 0.024$). The PPG after TIPS had no significant influence on median survival times in both groups (Table 2). Mortality was not significantly increased in patients aged > 65 years (Table

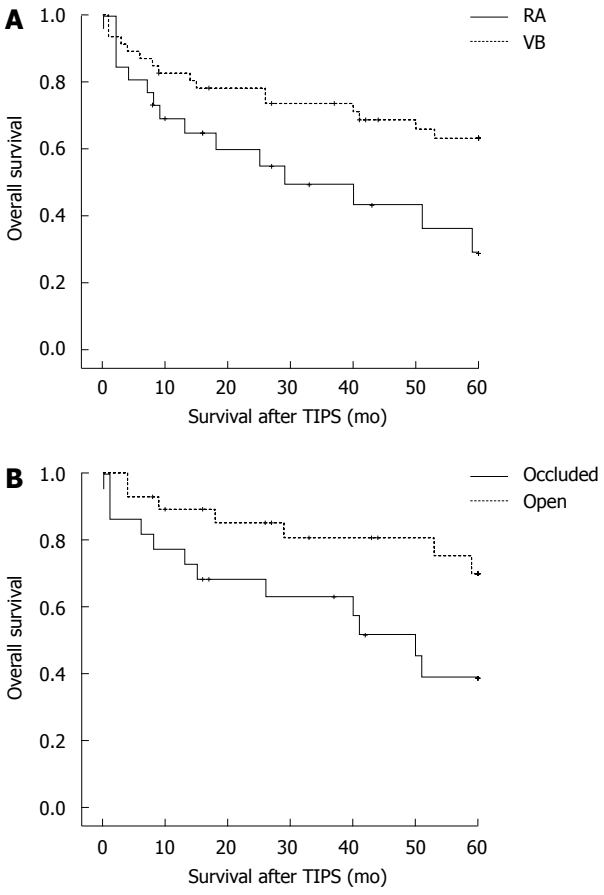


Figure 2 Kaplan-Meier survival analysis of patients after transjugular intrahepatic portosystemic shunt placement. A: In patients with initial ascites as indication for transjugular intrahepatic portosystemic stent (TIPS), survival is significantly shorter than that in patients with variceal bleeding [refractory ascites (RA) *vs* variceal bleeding (VB), log rank test $P = 0.009$]; B: In patients with occluded stent at first fluorographic control, survival is significantly shorter than that in patients with open stent (occluded *vs* open, log rank test $P = 0.027$).

2). For those patients having a MELD score greater than 10, the median survival was significantly shorter than for those with a MELD score less than or equal to 10 (35.3 mo *vs* 52.2 mo, $P = 0.001$).

In the Cox regression model, only stent occlusion at first control ($P = 0.022$), ascites prior to TIPS ($P = 0.043$), CHILD stage ($P = 0.015$) and MELD score ($P = 0.004$) were independent prognostic factors of survival. In contrast, anticoagulation management ($P = 0.097$), the porto-systemic pressure gradient ($P = 0.460$) and rebleeding episodes ($P = 0.765$) had no significant effect on the overall survival.

We further performed a subgroup analysis using the Kaplan-Meier method in terms of survival of the two groups considering the independent risk factors by Cox regression model analysis such as age, stent patency at first control, CHILD and MELD scores.

When survival was analyzed based on MELD scores (Figure 3A and B) we found that patients with VB had a statistically improved survival over those with RA (MELD score < 10 *vs* $\geqslant 10$, log rank $P = 0.001$).

Stratification by CHILD stages B and C or age > 65 years demonstrated that patients in the VB group had a significantly improved long-term survival compared with those in the RA group (log rank test $P = 0.021$ each) (Figure 3C and D).

Due to limited patient numbers the overall survival in patients with stent occlusion at first control did not differ significantly in both groups (Figure 3E, log rank test $P = 0.289$).

DISCUSSION

Since its introduction in the 1980s, the TIPS procedure has played a major role in the management of portal hypertension[9,24,40-43]. In the present study, shunt insertion was completed successfully in 81 patients (80% of patients scheduled). The baseline characteristics show the heterogeneous patient population at our hospital, the distribution of underlying diseases is typical for western countries[44,45] (Table 1).

Until recently, bare metal stents were the treatment of choice for establishing the TIPS tract. In contrast to the actual AASLD guidelines[9], in the United States[46] about 20% of all TIPS procedures still use uncovered TIPS stents.

Even though covered TIPS stents require fewer reinterventions, after a 12-mo-follow-up, the total procedure-related expenses were higher with covered TIPS stents due to their higher initial cost[43]. Further, a study by Bureau *et al*[25] in 2007 could not detect any survival benefit of covered *vs* uncovered stents. For these reasons we used non-coated TIPS stents during the study period of 1998 until 2008. Since this study was initiated at our institution, polytetrafluoroethylene-covered stents are now widely used, with the recent literature showing a significant improvement of primary patency up to 90% within 12 mo of application[25,28,47].

In agreement with Membreno *et al*[48], we show that in patients with TIPS due to VB, the overall long-term survival is significantly better than that in patients with TIPS due to RA (> 60 mo *vs* 29 mo, $P = 0.009$) (Figure 2A).

In the VB and RA groups of our study, the degree of reduction of the PPG following TIPS implantation was almost identical and there was no significant correlation with stent diameters. According to the literature, an adequate decompression of portosystemic hypertension can be achieved by 50% reduction of the initial pressure[49]. Other series describe a 20% reduction as sufficient and the PPG should be decreased and maintained under 12 mmHg[50]. In our study, the PPG was lowered post-procedurally at a recommended threshold of approximately 12 mmHg[24] (VB 11.8 ± 4.0 mmHg *vs* RA 11.7 ± 4.2 mmHg). Biecker *et al*[34] demonstrated in their study with 118 cirrhotic patients, that the initial decrease in the PPG after TIPS is a predictor of the rebleeding risk, but not of survival. Our study was not able, however, to confirm these findings.

In our patient cohort, the Cox multivariate regression analysis identified stent occlusion at first control as an

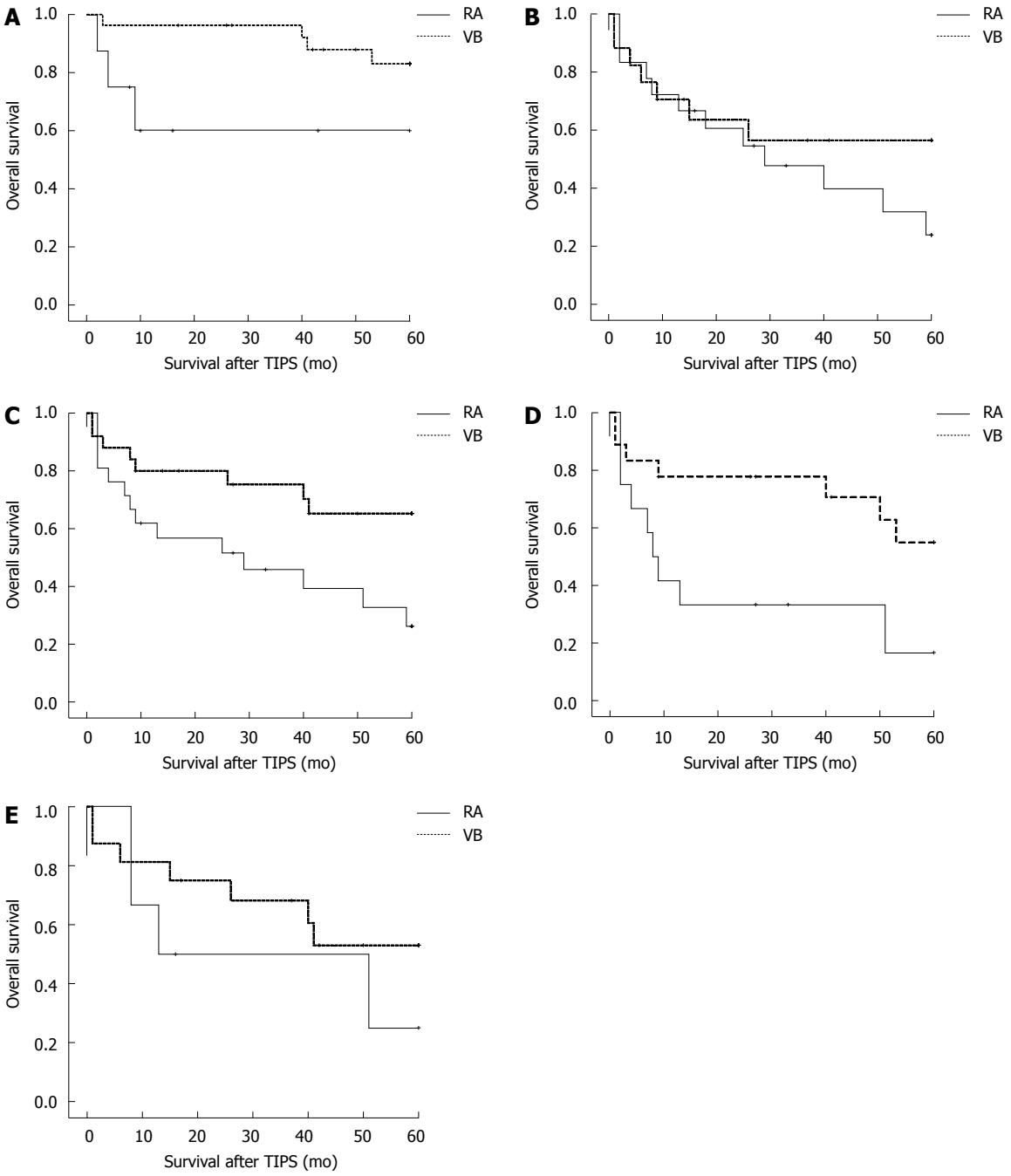


Figure 3 Kaplan-Meier survival analysis of patients after transjugular intrahepatic portosystemic shunt placement. A: Subgroup analysis with patients having a model for end-stage liver disease score (MELD) < 10: Significant difference in overall survival relating to indication [refractory ascites (RA) *vs* variceal bleeding (VB) group, log rank test $P = 0.031$]; B: Subgroup analysis with patients having a MELD score > 10: No significant difference in overall survival relating to indication (RA *vs* VB group, log rank test $P = 0.274$); C: Subgroup analysis with patients with CHILD B or C cirrhosis: Significant difference in overall survival relating to indication (RA *vs* VB group, log rank test $P = 0.021$); D: Subgroup analysis with patients age > 65 years: Significant difference in overall survival relating to indication (RA *vs* VB group, log rank test $P = 0.021$); E: Subgroup analysis with stent occlusion at first control: No significant difference in overall survival relating to indication (RA *vs* VB group, log rank test $P = 0.289$).

independent predictor of survival regardless of the indication for TIPS (Figure 2B and Figure 3E). Therefore, regular monitoring of the TIPS patients is highly recommended to provide early intervention when stenosis occurs[51]. In our institution, after successful TIPS insertion the first controls are conducted within 3 mo. Based on the results of the first interventional control (angiography), the following examinations are scheduled. Routinely colour Doppler ultrasound is used as a non-invasive device for monitoring the TIPS function.

Unsurprisingly, CHILD stage was an independent prognostic factor of survival ($P = 0.015$), probably due to the fact that the CHILD scoring system is a validated tool for assessing prognosis[39,52]. When survival was analyzed based on CHILD B or C, we found that patients with VB had a statistically improved survival over those

with RA (Figure 3C). Similar findings could be observed for patients being older than 65 years or having a MELD score < 10 leading to a significant overall survival relating to the indication for TIPS as displayed in Figure 3A, B and D.

These observations are consistent with those by Membreno *et al*[48]. The retrospective design and the use of uncovered stents as well as the relatively small sample size may introduce a certain bias. Nevertheless, our retrospective study emphasises several clinical aspects of portal hypertension in liver cirrhosis to be considered in conjunction with TIPS treatment.

In conclusion, TIPS is an established and safe nonsurgical method to decompress portal hypertension and to avoid its sequelae. RA prior to TIPS and stent occlusion at first control are independent predictors of survival in patients with bare metal TIPS shunts. This observation militates in favour of close follow-ups for patients with TIPS due to RA.

COMMENTS

Background

Liver cirrhosis is a common problem in gastroenterology. Various medical and interventional treatment options have been developed to manage the complications of portal hypertension. Minimal invasive placement of a transjugular intrahepatic portosystemic shunt (TIPS) is now widely used to lower the portosystemic pressure gradient.

Research frontiers

The authors undertook a retrospective study to work out the clinical outcome and predictors of survival after TIPS insertion with bare metal stents.

Innovations and breakthroughs

Refractory ascites (RA), stent occlusion at first control, initial CHILD stage and model for end-stage liver disease score were identified as independent predictors of survival in cirrhotic patients after TIPS implantation.

Applications

By understanding, which risk factors can influence survival in cirrhotic patients scheduled for TIPS insertion, the authors contribute to a better knowledge of this common clinical scenario. This may lead to a better risk stratification for the indication of TIPS insertion.

Terminology

The TIPS procedure decompresses the portosystemic pressure by establishing a "short cut" between the portal vein and the caval venous system.

Peer review

This is a retrospective study with the major objective to observe the role of stenosis and occlusion rates of uncovered stents on the survival of cirrhotic patients with TIPS inserted for variceal bleeding or RA.

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BRIEF ARTICLE

Effectiveness of infliximab after adalimumab failure in Crohn's disease

María Chaparro, Montserrat Andreu, Manuel Barreiro-de Acosta, Esther García-Planella, Elena Ricart, Eugeni Domènech, María Esteve, Olga Merino, Pilar Nos, Mireia Peñalva, Javier P Gisbert

María Chaparro, Javier P Gisbert, Department of Gastroenterology, Hospital Universitario de La Princesa, Instituto de Investigación Princesa (IP) and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), 28006 Madrid, Spain
María Chaparro, Americio 17, portal E 2ºC, 28021 Madrid
Montserrat Andreu, Department of Gastroenterology, Hospital del Mar, 08003 Barcelona, Spain
Manuel Barreiro-de Acosta, Department of Gastroenterology, Hospital Clínico Universitario de Santiago, 15706 Santiago de Compostela, Spain
Esther García-Planella, Department of Gastroenterology, Hospital Santa Creu i Sant Pau, 08025 Barcelona, Spain
Elena Ricart, Department of Gastroenterology, Hospital Clínic, Barcelona, and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, 08916 Badalona, Spain
Eugeni Domènech, Department of Gastroenterology, Hospital Germans Trias i Pujol and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), 08916 Badalona, Spain
María Esteve, Department of Gastroenterology, Hospital Mutua de Terrassa, 08221 Barcelona, Spain
Olga Merino, Department of Gastroenterology, Hospital de Cruces, 48903 Bilbao, Spain
Pilar Nos, Department of Gastroenterology, Hospital Universitario La Fe and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), 46009 Valencia, Spain
Mireia Peñalva, Department of Gastroenterology, Hospital de Bellvitge, 08907 Barcelona, Spain
Author contributions: Chaparro M and Gisbert JP were involved in the study concept and design, the statistical analysis and writing of the manuscript; Chaparro M, Andreu M, Barreiro-de Acosta M, García-Planella E, Ricart E, Domènech E, Esteve M, Merino O, Nos P, Peñalva M and Gisbert JP were all involved in acquisition of data and reviewed the manuscript critically; and all the authors approved the final version of the manuscript.
Correspondence to: María Chaparro, MD, Americio 17, portal E 2ºC, 28021 Madrid, Spain. mariachs2005@gmail.com
Telephone: +34-91-3093911 Fax: +34-91-4022299
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Abstract

AIM: To evaluate the effectiveness of infliximab as a second-line therapy in Crohn's disease patients after adalimumab failure.

METHODS: A historical cohort study in a community-based gastroenterology practice evaluated Crohn's disease patients treated with infliximab (induction plus maintenance) after adalimumab failure. Patients were identified using a large Spanish database (ENEIDA).

RESULTS: We included 15 Crohn's disease patients who received infliximab after adalimumab failure. Five patients discontinued adalimumab due to loss of response, 3 due to adverse events and 7 due to partial response. After infliximab therapy was started, all patients who had interrupted adalimumab due to loss of efficacy regained response. All patients who discontinued adalimumab due to adverse events responded to infliximab and maintained this response; one of these patients had an uneventful course on infliximab, but 2 developed adverse events. None of the 7 patients who interrupted adalimumab due to partial response reached remission with infliximab.

CONCLUSION: Switching from adalimumab to infliximab may be useful in patients who develop adverse effects or loss of response, however, the benefit of infliximab in primary nonresponders was not established.

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Key words: Adalimumab; Biologics; Crohn's disease; Infliximab; Switch

Peer reviewer: Dr. Grigoriy E Gurvits, Department of Gastroenterology, New York University School of Medicine and Medical Center, 530 First Avenue, SKI-9N New York, NY 10016, United States

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INTRODUCTION

Crohn's disease (CD) is a chronic, relapsing, transmural inflammation of the gastrointestinal tract that affects mainly young patients, and results in a considerably decreased quality of life[1]. There is no medical or surgical cure for CD, and the goal of the existing therapeutic modalities is to induce and maintain remission[2]. Since tumor necrosis factor (TNF)-α has a pivotal role in the pathogenesis of CD[3], the introduction of antibodies against TNF-α has created new perspectives for the management of this disease[4,5].

Infliximab is a murine, chimeric, monoclonal immunoglobulin G1 antibody against TNF-α which was approved for CD treatment ten years ago. Infliximab is effective in inducing and maintaining remission in patients with moderate to severe CD and in patients who have failed conventional non-biologic therapy[6]. However, one-third of these patients do not respond to induction therapy. In addition, a subset of patients who initially respond to infliximab will discontinue therapy due to loss of response or intolerance[7,8].

Adalimumab is a recombinant fully human immunoglobulin G1 monoclonal antibody against TNF-α. Randomized controlled trials have demonstrated the efficacy of adalimumab for induction and maintenance of remission in patients naïve to anti-TNF therapy[9-11]. There are also several studies providing data on the efficacy of adalimumab after infliximab failure[12-16]. The CHARM trial[9] evaluated adalimumab as a maintenance therapy in patients after infliximab failure as part of a subgroup analysis. The GAIN study[16], a randomized placebo-controlled trial, demonstrated that adalimumab therapy is effective in inducing remission in patients with active CD who initially responded to infliximab, but then lost response or became intolerant to the drug.

Since adalimumab was approved in 2007 for use in CD, it can be prescribed as a first-line therapy, and similar to infliximab, one-third of patients do not respond to this drug and a proportion of patients can lose efficacy or become intolerant over time[12,17,18]. In clinical practice, patients who do not respond to infliximab, who lose response, or become intolerant may be prescribed adalimumab in an attempt to regain response, but to the best of our knowledge, there are no published data on the effectiveness of infliximab after adalimumab failure. Therefore, the aim of this study was to evaluate the effectiveness and safety of infliximab after adalimumab failure in CD patients.

MATERIALS AND METHODS

Study subjects

Patients who received infliximab for CD after adalimumab failure at a community-based gastroenterology practice were evaluated in a historical cohort study. They were identified using a large Spanish database (ENEIDA), promoted by the Spanish Working Group in Crohn's and Colitis (GETECCU), including patients with inflammatory bowel disease. The database prospectively records the use, effectiveness and adverse events of immunomodulators and anti-TNF therapy. The database at the time of the study included 10752 patients, of whom 5467 had CD. Patients were excluded from the study if adalimumab or infliximab was initiated for treatment of a disease other than CD.

Data collection

Data collected included: sex, age, smoking status, age at diagnosis, location of disease, disease behavior (inflammatory, stenosing or fistulizing), perianal disease, concurrent use of immunomodulators, indication of anti-TNF therapy, data regarding adalimumab therapy (start date for adalimumab therapy, initial response, loss of response, date of loss of response, dose escalation of adalimumab after loss of response, response to escalated dose, loss of response to escalated dose, date of loss of response to escalated dose, adverse events with standard and with escalated treatment), reasons for discontinuation of adalimumab therapy, data regarding infliximab therapy (start date for infliximab therapy, initial response, loss of response, date of loss of response, dose escalation of infliximab after loss of response, response to escalated dose, loss of response to escalated dose, date of loss of response to escalated dose, adverse events with standard and with escalated treatment) and reasons for discontinuation of infliximab treatment. Individual charts were reviewed to obtain all data.

Definitions

Dose escalation: Dose escalation of adalimumab was defined as a decrease in the interval of administration from every-other-week to every-week. In the case of infliximab, dose escalation was defined either as an increase in infliximab dose, e.g., from 5 to 10 mg/kg, or a decrease in infliximab infusion interval, e.g., from every 8-wk to every 4-wk, or both a dose increase and an interval decrease.

Evaluation of response: For luminal disease, response to adalimumab and infliximab was evaluated using the Harvey-Bradshaw index (HBI)[19] four weeks after the first dose. Partial response was defined as a decrease in the HBI of more than 3 points. Remission was defined as a HBI below or equal to 4 without steroids. In perianal CD, complete response was defined as closure of all fistulas and partial response as a 50% or more reduction in the number of draining fistulas.

Loss of efficacy: Loss of efficacy was defined as impairment in patient's symptoms coupled with endoscopic, radiographic, and/or serologic (elevated C-reactive protein) evidence of inflammation that made the physician escalate the dose of treatment or change to other drug.

Disease behavior and location: Disease behavior was categorized based on the Montreal classification as: (1) inflammatory or CD without fistulizing or stricturing complications; (2) stricturing disease was defined as the presence of clinical symptoms of partial or complete obstruction with fixed narrowing and/or narrowing with proximal dilatation; and (3) fistulizing, which included the presence of enteric fistulas, intraabdominal abscesses, or bowel perforation. The location of disease was established by identifying macroscopic evidence of CD in any part of the gastrointestinal tract. Possible categories of disease location included the ileum, colon, ileum and colon, upper gastrointestinal tract, and perianal/perineal area.

Concomitant immunomodulators: Concomitant immunosuppressive treatment was considered if a patient had been on immunomodulators for at least the first 6 mo after starting the anti-TNF therapy.

Smoking history: Smoking was defined as the consumption of at least 1 cigarette daily for a period of at least 3 mo prior to study entry.

Statistical analysis

mean ± SD was calculated for continuous variables. Percentages and 95% confidence intervals were provided for categorical variables.

RESULTS

We included 15 CD patients who received infliximab after adalimumab failure. The main characteristics of the study population are summarized in Table 1. Mean time from diagnosis to adalimumab treatment was 69 mo and the median time of adalimumab treatment was 5 mo (range: 2-19 mo). Four patients had stricturing CD. Two of these patients discontinued treatment with adalimumab due to partial response, one patient had an initial partial response but lost this response, and the other patient was in remission but experienced adverse events which led to the interruption of adalimumab. The two patients who had a partial response achieved a partial response after switching to infliximab and the patient who lost the partial response achieved a partial response with infliximab. The patient who had adverse events tolerated infliximab without secondary effects. Four patients received immunosuppressants concomitantly with adalimumab and seven patients were on immunosuppressants previously and maintained this therapy when they started adalimumab.

Adalimumab discontinuation due to loss of efficacy

Five patients discontinued adalimumab due to loss of efficacy. Three were women, 3 had CD of the ileum, 4 showed inflammatory behavior and one perianal disease. Two of these patients had extraintestinal manifestations and 2 were on concomitant immunomodulators (azathioprine).

Median time to loss of efficacy of adalimumab was 6.2 mo (range: 3-9 mo). After loss of efficacy, the dose of adalimumab was escalated in 3 of these patients, but only 1 responded to this treatment strategy (Table 2).

All patients who discontinued adalimumab due to loss of efficacy regained response after switching to infliximab (3 reached remission and 2 had a partial response) (Table 2).

Table 1 Characteristics of study patients *n* (%)

Gender (female %)	10 (67)
Median age (yr)	33
Time of evolution to adalimumab therapy (mo)	69
Location	
L1	7 (47)
L2	1 (6)
L3	7 (47)
Behavior (%)	
Inflammatory	10 (67)
Stricturing	4 (27)
Fistulizing	1 (6)
Perianal disease	4 (27)
Smoking habit	3 (21)
Previous surgical resection	7 (47)
Concomitant immunosuppressants	11 (71)
Reason for discontinuation of adalimumab	
Partial response	7 (47)
Loss of efficacy	5 (33)
Adverse events	3 (20)

L1: Ileum; L2: Colon; L3: Ileum and colon.

Adalimumab discontinuation due to lack of response

Seven patients discontinued adalimumab due to partial response. Four were women, 5 had CD of the ileocolic region, 6 showed inflammatory behavior and 2 perianal disease. The dose of adalimumab was escalated in 3 of these patients with the aim of reaching remission, but without improvement in response (Table 2).

None of the 7 patients who interrupted adalimumab due to partial response reached remission with infliximab: 5 (71%) maintained partial response and 2 (29%) lost partial response (Table 2).

Adalimumab discontinuation due to adverse events

Three patients discontinued adalimumab due to adverse events: 1 had facial edema, 1 an injection site reaction and 1 had dizziness. All of these patients were female, 2 had CD of the ileum, 2 showed stricturing behavior, 1 perianal disease and 2 were on concomitant immunomodulators (azathioprine). Two of these patients were in remission and 1 had a partial response at the time of adalimumab discontinuation due to adverse events. All patients maintained the response they had with adalimumab after switching to infliximab (patients in remission maintained

Table 2 Responses to the switch from adalimumab to infliximab

Reason for ADA discontinuation	Indication for anti-TNF	Initial response to ADA	Final response to ADA	ADA escalation	Initial response to ADA escalation	Final response to ADA escalation	Initial response to IFX	AE with IFX
Loss of response	Perianal	Partial response	No response	No	NA	NA	Remission	No
Loss of response	Luminal	Remission	No response	No	NA	NA	Remission	No
Loss of response	Luminal	Partial response	No response	Yes	Partial response	No response	Remission	No
Loss of response	Luminal	Partial response	Partial response	Yes	No response	NA	Partial response	No
Loss of response	Luminal	Partial response	No response	Yes	No response	NA	Partial response	No
Partial response	Luminal	Partial response	Partial response	No	NA	NA	Partial response	No
Partial response	Perianal	Partial response	No response	Yes	Partial response	Partial response	Partial response	No
Partial response	Luminal	Partial response	Partial response	No	NA	NA	Partial response	No
Partial response	Luminal	Partial response	Partial response	No	NA	NA	Partial response	No
Partial response	Luminal	Partial response	Partial response	Yes	Partial response	Partial response	No response	No
Partial response	Luminal	Partial response	Partial response	Yes	Partial response	Partial response	No response	No
Partial response	Perianal	Partial response	Partial response	No	NA	NA	Partial response	No
Adverse events	Luminal	Remission	Remission	No	NA	NA	Remission	No
Adverse events	Perianal	Partial response	Partial response	No	NA	NA	Partial response	Yes
Adverse events	Luminal	Remission	Remission	No	NA	NA	Remission	Yes

ADA: Adalimumab; TNF: Tumour necrosis factor; IFX: Infliximab; NA: Not applicable; AE: Adverse events.

remission with infliximab and the single patient with partial response had a partial response to infliximab). One of these patients received infliximab uneventfully, 1 had a delayed hypersensitivity reaction controlled by premedication with steroids before infliximab infusion, and the third patient interrupted infliximab due to facial edema (the same adverse event that forced the discontinuation of adalimumab).

DISCUSSION

The treatment of CD has evolved over the past decade with the introduction of anti-TNF agents. However, some patients do not respond or show suboptimal response to these drugs. Furthermore, patients who respond initially may lose efficacy over time or develop adverse events, which sometimes forces them to discontinue treatment. In these different scenarios, switching from one anti-TNF-α to another could represent an option for CD patients who fail the first anti-TNF drug.

The findings of the present observational study suggest that the probability of achieving clinical response after switching from adalimumab to infliximab may be higher in patients who discontinue adalimumab due to loss of efficacy or adverse events; as compared to those switching due to primary failure with adalimumab. In fact, patients who did not reach remission with adalimumab had no response to infliximab, whereas a relatively high proportion of patients showed a satisfactory response after discontinuing adalimumab due to loss of response or adverse events.

These observations seem to be in agreement with some of the published reports focusing on the effectiveness of adalimumab after infliximab failure[11,12,16,17]. In this respect, we have evidence that after loss of efficacy or intolerance to infliximab, adalimumab can be effective. However, to the best of our knowledge, this is the first study evaluating the effectiveness of infliximab after adalimumab failure.

Both infliximab and adalimumab are monoclonal antibodies and can be recognized by the human immune system as foreign antigens which respond by creating their own antibodies to different sites on the molecule[7,20,21]. In the case of infliximab, the development of antibodies to infliximab and, as a consequence, low trough concentration of the drug, have been implicated as predisposing factors for infliximab treatment failure[21-24]. The presence of antibodies to infliximab has been associated with the development of hypersensitivity reactions (infusional or delayed), while low trough concentration of infliximab has shown a high correlation with loss of response to treatment[7,21,24,25].

Although fully human, adalimumab is not devoid of immunogenicity. Antibodies to adalimumab have been reported in 2.6%-38% of patients treated for CD and rheumatoid arthritis[11,26,27]. In patients with rheumatoid arthritis, antibodies to adalimumab have been associated with low adalimumab trough serum concentration and decreased clinical response[26]. Karmiris *et al*[20] in a recently published study on CD patients treated with adalimumab after infliximab failure, found that 9% of patients developed antibodies to adalimumab, and that patients who developed antibodies to adalimumab frequently had low trough serum concentrations. They also reported that adalimumab trough serum concentration was lower throughout the follow-up period in patients who had to discontinue treatment due to loss of efficacy[20]. These findings suggest that the role of immunogenicity in the loss of response and in the development of adverse events following adalimumab treatment may be similar to that previously described with infliximab.

There is a lack of data on the development of adverse events with an anti-TNF drug in the subgroup of patients that had discontinued other anti-TNF drugs due to this reason, as the published studies provide this information globally irrespective of the type of failure (loss of efficacy or adverse events)[7,11,16]. The occurrence of immunoallergic reactions has been related to the for-

mation of antibodies against the anti-TNF drug and, in this respect, Karmiris *et al*[20] found that the presence of antibodies to infliximab before initiation of adalimumab therapy was not associated with a higher incidence of antibodies against adalimumab. There are no data on the development of antibodies to infliximab in patients with previous antibodies to adalimumab, but we would expect that they were not increased based on the findings of the previously mentioned study[20]. In our study, among the 3 patients who discontinued adalimumab due to adverse events, 2 had adverse events with infliximab and 1 of them had to interrupt infliximab due to the same adverse event which had developed with adalimumab. Considering that antibodies to one anti-TNF drug do not seem to be increased in patients with antibodies to other anti-TNF drug, our findings could be explained by the existence of cross reactions between antibodies to adalimumab and infliximab molecules, but this hypothesis has not been proved.

Finally, we found no benefit after switching from adalimumab to infliximab in patients who discontinued treatment due to partial response to adalimumab. The lack of efficacy of the anti-TNF agent in these patients could be due to particular disease characteristics in which TNF-α does not play a pivotal role[23]. In the adjunctive catheter-directed thrombolysis trial performed in rheumatoid arthritis patients, the authors found that an incomplete response to anti-TNF therapy in the population included in the study was not related to an insufficient concentration of the drug or to the presence of antibodies against it, concluding that other pro-inflammatory molecules different from TNF-α could play a main role in these patients.

One limitation of our study is the small sample size. Although our results are original, since there are no published data on the efficacy and safety of infliximab after adalimumab failure in CD patients, studies with a larger sample size are needed to establish the benefit of switching to another anti-TNF agent after one anti-TNF agent has failed.

In conclusion, the results of the present study suggest that CD patients may be successfully treated with infliximab after adalimumab failure, specifically those withdrawing for loss of efficacy or adverse events. Conversely, in patients discontinuing adalimumab due to lack of response, the efficacy of infliximab was not established and other drugs with different targets might offer a greater chance of therapeutic success.

COMMENTS

Background

There is no medical or surgical cure for Crohn's disease (CD), and the goal of the existing therapeutic modalities is to induce and maintain remission. Infliximab and adalimumab are monoclonal antibodies against tumor necrosis factor (TNF-α). However, one third of patients can lose response or become intolerant to these drugs.

Research frontiers

Studies are being performed in order to identify which are the factors responsible of the loss of efficacy or intolerance to anti-TNF drugs.

Innovations and breakthroughs

In clinical practice, patients who do not respond to infliximab, who lose response or become intolerant may be prescribed adalimumab in an attempt to regain response. Data regarding the outcome of patients who received infliximab after adalimumab failure are scarce. This study assesses the effectiveness of infliximab after adalimumab failure in CD patients.

Applications

The results of the present study suggest that CD patients may be successfully treated with infliximab after adalimumab failure, specifically those withdrawing due to loss of efficacy or adverse events. Conversely, in patients discontinuing adalimumab due to lack of response, the efficacy of infliximab has not been established and other drugs with different targets might offer a greater chance of therapeutic success.

Peer review

This is a good study in which the authors valuated the effectiveness of infliximab as a second-line therapy in CD patients after adalimumab failure. The result is interesting and suggested that CD patients may be successfully treated with infliximab after adalimumab failure.

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BRIEF ARTICLE

Contrast-enhanced ultrasound evaluation of hepatic microvascular changes in liver diseases

Francesco Ridolfi, Teresa Abbattista, Paolo Busilacchi, Eugenio Brunelli

Francesco Ridolfi, Eugenio Brunelli, Division of Gastroenterology, Principe di Piemonte Hospital, 60019 Senigallia, AN, Italy
Teresa Abbattista, Division of Radiology, Principe di Piemonte Hospital, 60019 Senigallia, AN, Italy
Paolo Busilacchi, SIUMB School of Ultrasound of Ancona, Principe di Piemonte Hospital, 60019 Senigallia, AN, Italy
Author contributions: Ridolfi F and Abbattista T designed the study, performed the examinations and wrote the manuscript; and Busilacchi P and Brunelli E provided the analytical tools and edited the manuscript.
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Correspondence to: Francesco Ridolfi, MD, PhD, Division of Gastroenterology, Principe di Piemonte Hospital, Via Cellini 1, 60019 Senigallia, AN, Italy. francescoridolfi71@gmail.com
Telephone: +39-71-79092606 Fax: +39-71-79092604
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Abstract

AIM: To assess if software assisted-contrast-enhanced ultrasonography (CEUS) provides reproducible perfusion parameters of hepatic parenchyma in patients affected by chronic liver disease.

METHODS: Forty patients with chronic viral liver disease, with (n = 20) or without (n = 20) cirrhosis, and 10 healthy subjects underwent CEUS and video recordings of each examination were then analysed with Esaote's Qontrast software. CEUS dedicated software Qontrast was used to determine peak (the maximum signal intensity), time to peak (TTP), region of blood value (RBV) proportional to the area under the time-intensity curve, mean transit time (MTT) measured in seconds and region of blood flow (RBF).

RESULTS: Qontrast-assisted CEUS parameters displayed high inter-observer reproducibility (κ coefficients of 0.87 for MTT and 0.90 TTP). When the region of interest included a main hepatic vein, Qontrast-calculated TTP was significantly shorter in cirrhotic patients (*vs* non-cirrhotics and healthy subjects) (71.0 ± 11.3 s *vs* 82.4 ± 15.6 s, 86.3 ± 20.3 s, $P < 0.05$). MTTs in the patients with liver cirrhosis were significantly shorter than those of controls (111.9 ± 22.0 s *vs* 139.4 ± 39.8 s, $P < 0.05$), but there was no significant difference between the cirrhotic and non-cirrhotic groups (111.9 ± 22.0 s *vs* 110.3 ± 14.6 s). Peak enhancement in the patients with liver cirrhosis was also higher than that observed in controls (23.9 ± 5.9 *vs* 18.9 ± 7.1, P = 0.05). There were no significant intergroup differences in the RBVs and RBFs.

CONCLUSION: Qontrast-assisted CEUS revealed reproducible differences in liver perfusion parameters during the development of hepatic fibrogenesis.

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Key words: Contrast enhanced ultrasound; Cirrhosis; Hepatitis; Liver perfusion; Hepatic microcirculation

Peer reviewers: Herwig R Cerwenka, Professor, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, Graz A-8036, Austria; Soeren Rafaelsen, MD, Department of Radiology, Vejle Hospital, Vejle 7100, Denmark; Fikri M Abu-Zidan, Professor, Department of Surgery, Faculty of Medicine, UAE University, PO Box 17666, Al-Ain, United Arab Emirates

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INTRODUCTION

Changes in hepatic perfusion are a key feature of cirrhosis[1]. Capillarization of the sinusoids with loss of

endothelial fenestration and increased tone of activated hepatic stellate cells (HSCs) increase the mechanical resistance to portal blood flow and augment hepatic vascular tone, leading to portal hypertension[2]. Alterations involving the microvascular bed of the liver are already evident during the precirrhotic stages of hepatic fibrogenesis. The ongoing liver damage induces the overexpression of angiogenic growth factors (such as vascular endothelial growth factor or platelet-derived growth factor)[3], which promote the persistence of inflammatory changes and fibrogenic tissue repair, facilitating the development of shunts between branches of the portal vein, the hepatic veins, and the hepatic artery within the newly formed fibrotic septa[2,4].

Computed tomography (CT) and magnetic resonance imaging (MRI) have both been used for quantitative and qualitative assessment of parenchymal perfusion in cirrhotic livers. CT studies revealed increased hepatic arterial flow and decreased mean transit times (MTT), as compared with values observed in control subjects[5]. In contrast, MRI with low molecular weight contrast material revealed increases in the MTT[6]. Use of these methods in clinical practice is limited, however, by the intensive post-processing required to obtain perfusion data, high costs, and the lack of standardized examination protocols[7].

B-mode ultrasonography with Doppler study of hepatic vessels is often the first-line imaging study for the work-up of patients with diffuse liver disease. The Doppler technique is used mainly to measure flow in macroscopic vessels and is not strictly related to the microcirculation of the liver[8]. Contrast-enhanced ultrasonography (CEUS) is performed after intravenous administration of a suspension of gas-filled microbubbles, which remain entirely within the intravascular space and thus act as a blood pool tracer. CEUS studies of perfusion in the liver parenchyma have focused mainly on the measurement of contrast-medium transit times (from the portal vein to the hepatic veins), which have proved to be significantly shorter in patients with cirrhosis (compared with non-cirrhotic patients with chronic liver disease)[9-11]. CEUS has also documented increased regional perfusion of the hepatic parenchyma in cirrhotic patients (compared with healthy subjects), and this increase displayed correlation with the degree of liver failure[12].

A major shortcoming of CEUS is its user-dependency. Qontrast™ (Esaote S.p.a., Florence, Italy) is a post-processing computational tool, which can be used with CEUS to obtain objective, quantitative parameters of microvascular damage in various organs, including the liver.

We examined patients with chronic liver disease using CEUS with Qontrast analysis of hepatic parenchymal perfusion. The aims of this study were to assess the reliability and reproducibility of software assisted-CEUS studies of hepatic parenchymal perfusion and to evaluate if the analysis of the parameters obtained could help to understand vascular changes developing during liver fibrogenesis.

MATERIALS AND METHODS

The study protocol, which conformed to the guidelines outlined in the 1975 Declaration of Helsinki, was pre-approved by the institutional ethics committee. Written informed consent was obtained from all participants.

Study populations

Participants were enrolled between March 2007 and December 2010. They included 10 control patients with no liver disease (as documented by self-reported history and blood chemistry data obtained during screening) and 40 patients consecutively seen by our staff for chronic viral liver disease. At the time of enrollment, all of these patients had been positive for hepatitis B surface antigen and/or anti-hepatitis C virus antibodies for at least 6 mo.

All participants underwent complete physical examinations, laboratory tests, and standard B-mode abdominal ultrasonography. Candidates were excluded if they presented any of the following: (1) sonographic evidence of focal liver lesions; (2) history of alcohol consumption of $\geqslant$ 20 g/d; and (3) current use of medication known to affect the intra- or extra-hepatic circulation.

The patients with chronic liver disease included 20 patients in whom the absence of cirrhosis had been confirmed by liver biopsy (non-cirrhotic group) and 20 others (cirrhotic group) with cirrhosis diagnosed by liver biopsy or on the basis of commonly accepted clinical criteria. The latter included a history of chronic liver disease together with portal hypertension manifested by two or more of the following: (1) endoscopic evidence of esophageal or gastric varices and/or portal hypertensive gastropathy; (2) hypersplenism (reflected by a white blood cell count of $< 3500/mm^3$ and/or a platelet count of $< 100\,000\ /mm^3$); (3) sonographic evidence of ascites; and (4) a hepatic venous pressure gradient (HVPG) of $\geqslant$ 12 mmHg[13,14]. The severity of the cirrhosis was rated with the Child-Pugh[15] and model for end-stage liver disease (MELD)[16] systems.

The liver biopsies used for patient classification had all been performed under sonographic guidance 1-3 mo prior to enrollment (mean: 1.8 mo; median: 1 mo). For the purposes of this study, all slides were independently reviewed by a single experienced liver pathologist, who was blinded to the patient's clinical data and the results of his/her hepatic ultrasound examination. This examiner rated the presence of inflammation and fibrosis in each case with the Metavir scoring system[17].

Ultrasound and CEUS analysis

All sonographic examinations were carried out by an experienced radiologist using a 3.5-MHz convex array transducer and a General Electric Logiq 9 scanner (Milwaukee, WI, United States) equipped with software for contrast imaging and color and power Doppler. Patients were examined after a fast of 6 h. After a standard B-mode scan, the second phase of the examination started, during which we evaluated perfusion within the hepatic parenchyma using a sonographic contrast agent composed of sulfur hexafluoride-filled microbubbles (Sonovue®, Bracco Spa, Milan, Italy). The probe was placed over the middle hepatic vein (at least 3 cm from the confluence)

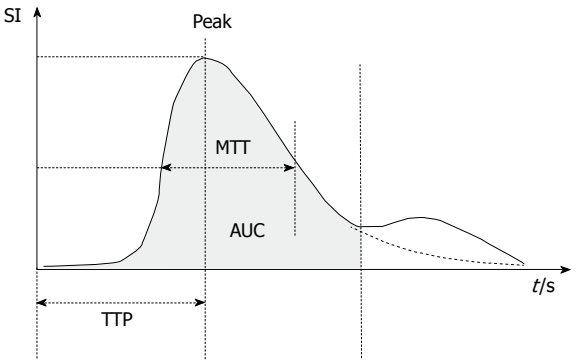


Figure 1 Main parameters extrapolated by Qontrast analysis of the time-intensity curve obtained by liver perfusion with an ultrasound contrast agent. SI: Signal intensity; TTP: Time to peak; MTT: Mean transit time; AUC: Area under the curve.

and surrounding tissue, where heartbeat artifacts were negligible. If an acceptable signal could not be obtained from this vein due to abdominal gas, the scan was made over the left or right hepatic vein. Tissue enhancement of these areas was recorded in a digital video format from 20 s before (baseline signal) to 130 s after the injection of a 2.5 mL bolus of Sonovue. Sonovue was injected manually into the antecubital vein at the rate of 1 mL/s, and the line was flushed with a 2.5-mL bolus of normal saline delivered at the same rate. Patients were instructed to breath gently during the procedure to minimize movement-related artifacts. Vital signs were monitored for 1 h after the examination, and patients were interviewed by phone 48 h after discharge to identify any adverse effects.

Video recordings were then analysed with the Qontrast software, which performs a full-map parametric analysis of perfusion within a selected set of frames in a specific region of interest (ROI). The loop of images is automatically processed after the tissue region and perfusion period have been defined; translational movements of the selected area can also be corrected. The area is then automatically aligned over all frames, and perfusion is analysed for points that continuously identify the moving tissue. Signal brightness is analysed separately at each point, and the optimal fitting curve is evaluated for each point.

In each patient, we evaluated two different ROIs. The first was a 25 cm^2 area of parenchyma that included one of the main hepatic veins and surrounding tissue; the second was a smaller area (5 cm^2) that included no major vessels. For each ROI, time-enhancement intensity curves were plotted with the Qontrast software, and the following parameters were generated (Figure 1): peak signal intensity (in dB) reached during the transit of the Sonovue bolus; time to peak (TTP) intensity, measured in seconds; regional blood volume (RBV), which is proportional to the area under the time-intensity curve; MTT (measured in seconds); and regional blood flow (RBF), which is the ratio of the RBV to MTT.

Intraobserver and interobserver agreement

We evaluated interobserver and intraobserver variation in measurements of the MTT and TTP during CEUS. For the former assessment, two observers (one experienced physician the other one in training) independently and blindly reviewed the video recording of each examination. For the latter analysis, each video was re-examined by one of the observers (still blind) 2-3 mo after the original review. Concordant measurements were those that differed by no more than ± 1 s.

Table 1 Clinical and biochemical characteristics of the three study groups (mean ± SD)

Group characteristics	Controls *n* = 10	Patients with chronic liver disease	
		Non-cirrhotic *n* = 20	Cirrhotic *n* = 20
Age (yr)	48.5 ± 14.4	48.9 ± 12.4	57.7 ± 11.4
Males, *n* (%)	4 (40)	12 (60)[c]	17 (85)[c]
Viral etiology (HBV/HCV)	0/0	2/18	4/16
Necroinflammatory score			
A0	-	3	0
A1	-	10	0
A2	-	7	3
A3	-	0	1
Fibrosis score			
F0	-	5	0
F1	-	6	0
F2	-	4	0
F3	-	5	0
F4	-	0	4
ALT (IU/L)	22.3 ± 5.2	113.8 ± 89.1[c]	76.9 ± 59.2[c]
Bilirubin (mg/dL)	0.6 ± 0.1	0.7 ± 0.4	1.5 ± 0.9[a]
Albumin (g/dL)	3.7 ± 0.3	4.1 ± 0.4	3 ± 0.8[a]
INR	1.1 ± 0.1	1 ± 0.1	1.2 ± 0.2[e]

[a]$P < 0.05$ *vs* other groups; [c]$P < 0.05$ *vs* control group; [e]$P < 0.05$ *vs* non-cirrhotic group. ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; INR: International normalized ratio; A0: No activity; A1: Mild activity; A2: Moderate activity; A3: Severe activity; F0: No fibrosis; F1: Portal fibrosis without septa; F2: Portal fibrosis with few septa; F3: Numerous septa without cirrhosis; F4: Cirrhosis.

Statistical analysis

Data were expressed as group mean ± SD. Differences between the three groups (cirrhotic patients, non-cirrhotic patients, and controls) were evaluated with the Kruskall-Wallis test. A post-hoc *t* test was then used to evaluate differences between each group of participants. Differences in the proportion of male or female patients were assessed with the χ^2 test. Kappa statistics were used to assess interobserver and intraobserver agreement in the calculation of the Qontrast parameters[18,19].

McGraw-Hill Primer Statistical Software (2nd edition, 1986) and MedCalc Statistical Software (version 11.6, 2011, Mariakerke, Belgium) were used for all analyses. Statistical significance was defined as $P < 0.05$.

RESULTS

The characteristics of the control group and the patients with chronic liver disease are shown in Table 1. Most of the patients with cirrhosis [diagnosed by liver biopsy (*n* =

Table 2 Contrast-enhanced ultrasonography parameters calculated with Qontrast software in the three study groups (mean ± SD)

Perfusion parameter	Controls		Non-cirrhotic patients		Cirrhotic patients	
	ROI with HV	ROI without HV	ROI with HV	ROI without HV	ROI with HV	ROI without HV
TTP (s)	86.3 ± 20.3	86.8 ± 24.9	82.4 ± 15.6	78.2 ± 12.0	71.0 ± 11.3[a]	73.7 ± 17.8
Peak (%)	18.9 ± 7.1	18.5 ± 7.8	25.9 ± 7.8[c]	23.1 ± 8.9	23.9 ± 5.9[c]	25.5 ± 6.6
RBV	2828.7 ± 1720	2827.4 ± 1642.1	3402.3 ± 1515.1	2734.9 ± 1327.5	2809.3 ± 1111.6	3031.9 ± 1000.2
RBF	21.4 ± 9.5	21.4 ± 10.5	29.3 ± 10.5	25.6 ± 11.1	27.2 ± 7.9	28.8 ± 8.1
MTT (s)	139.4 ± 39.8	128.4 ± 37.8	110.3 ± 14.6[c]	105.8 ± 17.1[c]	111.9 ± 22.0[c]	104.7 ± 24.4[c]

MTT: Mean transit time; RBV: Regional blood volume; RBF: Regional blood flow; TTP: Time to peak; ROI: Region of interest; HV: Hepatic vein. [a]$P < 0.05$ *vs* other groups; [c]$P < 0.05$ *vs* control group; [c]$P = 0.05$ *vs* control group.

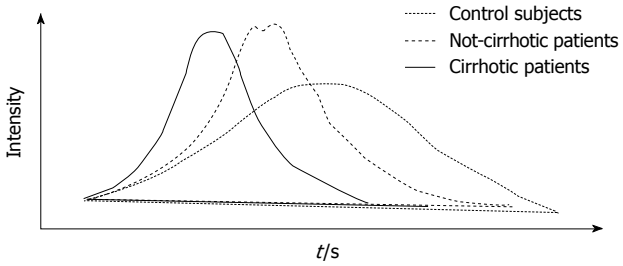


Figure 2 Schematic representation of time-intensity curves observed in the three study populations.

4) or on the basis of clinical criteria ($n = 16$)] were Child-Pugh class A ($n = 11$), but classes B and C were also represented (5 and 4 patients, respectively). The mean MELD score for the cirrhotic subgroup was 10.3 ± 3.9. Eleven of these patients had ascites, 2 had signs of hepatic encephalopathy, esophageal varices were found in 11, and HVPG more than 12 mmHg was evident in 3 patients. There were no significant age differences between the three groups, but males were significantly more common in the subgroups with chronic liver disease.

Ultrasound and CEUS studies were successfully completed for all participants, and no adverse effects were observed during or after the procedure (as documented by phone interviews with patients).

Qontrast analysis of contrast-enhanced ultrasound of liver parenchyma

The perfusion parameters generated by Qontrast analysis of the two ROIs examined is shown in Table 2: the first containing a hepatic vein and surrounding tissue, the second containing parenchyma alone, with no major vessels. In both cases, the MTTs in the patients with liver diseases were significantly shorter than those of controls, but there was no significant difference between the cirrhotic and non-cirrhotic groups. Analysis of the ROIs containing a hepatic vein (middle in 46 cases, right in 3, left in 1) revealed that TTPs in cirrhotic patients were significantly shorter than those of controls and of non-cirrhotic patients ($P < 0.05$). Similar findings emerged when we analyzed data obtained for the smaller ROIs although the differences in this case were not statistically significant. Peak enhancement in the patients with liver disease was also higher than that observed in controls, but again, this difference was significant only when the area analyzed included a main hepatic vein ($P < 0.05$). There were no significant intergroup differences in the RBVs, regardless of which ROI was considered. As for the RBF, the values observed in the chronic liver disease groups were appreciably (but not significantly) increased over those of controls, probably as a result of the shorter MTTs in these patients. This difference was also seen when the hepatic vein was not included in the ROI.

Observer agreement

Intraobserver agreement was calculated for MTT and TTP. Full agreement was considered when the two different analyses differed no more than ± 1 s. According to this finding, the agreement in reviewing Qontrast analysis for MTT and TTP by the same examiner was considered almost perfect: κ coefficient for MTT of 0.84 [95% confident interval (CI): 0.796-0.902] and for TTP of 0.92 (95% CI: 0.892-0.946). More interesting, the interobserver agreement for MTT and TTP by two examiners was found also to be almost perfect: κ coefficients for MTT of 0.87 (95% CI: 0.826-0.916) and for TTP of 0.90 (95% CI: 0.867-0.935).

DISCUSSION

CEUS represents the natural continuation of standard B mode ultrasound and Doppler studies, which are widely used for the diagnosis and follow-up of chronic liver disease. Compared with CT and MRI, ultrasound offers important advantages in terms of availability, safety, repeatability, and costs. We found CEUS of liver parenchyma to be safe and effective since we obtained a good quality digital video from each examination without any adverse effect observed. One of its main shortcomings is that it is highly operator dependent. We found Qontrast-assisted CEUS analysis of parenchymal perfusion to be highly reproducible. Intraobserver agreement was excellent (κ coefficient of 0.92) and, more interesting, interobserver agreement was almost perfect (κ coefficient: 0.90). Post-processing analysis of digitally recorded CEUS findings may thus be useful for standardizing this approach and improving its reproducibility.

When analysis was restricted to ROIs containing no major vessels, the time-intensity curves in the healthy control group were generally flat with a late enhancement peak (86.8 ± 24.9 s) and long MTT (range: 91.4-192.1 s),

findings that probably reflect an extensive vascular bed characterized by slow, continuous flow (Figure 2). By contrast, in patients with liver disease peak enhancement was higher and tended to occur earlier, and this pattern was more evident as the severity of the liver disease increased. From a quantitative point of view, this trend was reflected by MTTs in cirrhotic patients that were significantly shorter than those of the patients without liver disease. The steeper curve is an expression of faster, more concentrated flow of a volume of blood similar to that found in a healthy liver (the total hepatic blood volume is no different from that observed in the control group, as demonstrated by the RBVs). This picture (i.e., shorter MTTs associated with the same RBV) is fully compatible with sinusoid capillarization and increased activated HSC tone, which occur during the progression of chronic liver disease and can lead to high-velocity blood flow through the liver. When one of the major hepatic veins was included in the ROI, the TTP also decreased significantly across the three groups (controls > non-cirrhotics > cirrhotics). This picture could reflect the development of intrahepatic shunts that permit portal veins and branches of hepatic artery to cross cirrhotic areas, leading directly or indirectly into the central venous compartment[20].

Qontrast analysis indeed revealed more substantial differences between our patient subgroups when one of the hepatic veins was included in the ROI. The significant TTP shortening observed under these conditions is probably related in large part to the increasing presence of intra-hepatic shunts between branches of portal and/or hepatic artery and the hepatic veins. This is also the basis of the shortened hepatic vein arrival times documented in our cirrhotic patients (and those of other studies)[10,11,20] and also in patients with malignant liver disease[21].

The main limitation of our study is the small number of patients examined. Definitive conclusions on value of Qontrast analysis of CEUS data in diagnosing cirrhosis will have to be based on studies in much larger populations. In any case, use of this software does appear to increase the reproducibility of CEUS findings, and this could be useful for standardizing CEUS protocols and enhancing the comparability of findings obtained by different groups (that is for example a major weakness of Doppler examinations[22]).

Our initial experience with Qontrast-assisted CEUS studies of liver perfusion revealed clear differences between cirrhotic and non-cirrhotic patients with chronic liver disease and may serve as an incentive for further investigations. The reproducibility of Qontrast-assisted CEUS (although some training and experience are essential for optimal results) and its broad availability make it suitable for repeat examinations. Liver perfusion by CEUS could thus represent a valuable "non fibrotic-non invasive" tool to evaluate liver disease severity and to monitor the progression of chronic liver diseases.

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COMMENTS

Background

Changes in hepatic perfusion are a key feature that parallels the process of liver fibrogenesis developing during chronic liver diseases. These changes could be used to evaluate liver disease severity during the clinical follow up of chronic liver diseases.

Research frontiers

Evaluation of liver disease severity by studies of perfusion in the liver parenchyma has been mainly performed by computer tomography or magnetic resonance imaging. These methods in clinical practice are limited, however, by the intensive post-processing required to obtain perfusion data, high costs, and the lack of standardized examination protocols. Contrast enhanced ultrasonography (CEUS) could represent the best way to assess liver perfusion due to its wide availability, low cost and safety of ultrasound.

Innovations and breakthroughs

Previous studies performed by CEUS to assess liver disease severity have been mainly focused on the measurement of contrast-medium transit times (mainly from the portal vein to the hepatic veins). A major shortcoming of CEUS is its user-dependency. In this study the authors performed a software-assisted CEUS: Qontrast™ (Esaote S.p.a., Florence, Italy) is a post-processing computational tool, which can be used with CEUS to obtain objective, quantitative parameters of microvascular damage in the liver. The authors demonstrated that software assisted CEUS studies of liver perfusion revealed clear differences between cirrhotic and non-cirrhotic patients with chronic liver disease. The reproducibility of Qontrast-assisted CEUS is quite high and could be useful for standardizing CEUS protocols and enhancing the comparability of findings obtained by different groups.

Applications

The authors' initial experience with Qontrast-assisted CEUS studies of liver perfusion may serve as an incentive for further investigations. It is believed that liver perfusion by CEUS could represent a valuable "non fibrotic-non invasive" tool to evaluate liver disease severity and to monitor the progression of chronic liver diseases.

Terminology

Liver perfusion: Alterations involving the microvascular bed of the liver are already evident during the pre-cirrhotic stages of hepatic fibrogenesis. Main features are capillarization of the sinusoids with loss of endothelial fenestration and increased tone of activated hepatic stellate cells leading to the increase of the mechanical resistance to portal blood flow and augment hepatic vascular tone. The development of shunts between branches of the portal vein, the hepatic veins, and the hepatic artery within the newly formed fibrotic septa are also a key event; CEUS: Performed after intravenous administration of a suspension of gas-filled microbubbles, which remain entirely within the intravascular space and thus act as a blood pool tracer. CEUS studies of perfusion can be applied to the liver to evaluate the times and the intensity of enhancement of liver parenchyma occurring after the contrast injection (i.e., the time-intensity curve of enhancement).

Peer review

This is a good pivotal study in which the authors found time to peak contrast enhancement was significantly shorter in cirrhotic patients and contrast transit time was significantly shorter in the patients with liver diseases than those of controls. These results indicate that this new non-invasive method of analyzing hepatic vein transit time is useful for the prediction of liver disease progression.

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BRIEF ARTICLE

High-definition colonoscopy with i-Scan: Better diagnosis for small polyps and flat adenomas

Pier Alberto Testoni, Chiara Notaristefano, Cristian Vailati, Milena Di Leo, Edi Viale

Pier Alberto Testoni, Chiara Notaristefano, Cristian Vailati, Milena Di Leo, Edi Viale, Division of Gastroenterology and Gastrointestinal Endoscopy, Vita-Salute San Raffaele University, Scientific Institute San Raffaele, 20132 Milan, Italy
Author contributions: Testoni PA was responsible for the conception and design; Notaristefano C, Di Leo M and Vailati C contributed to the analysis and interpretation of the data; Testoni PA and Notaristefano C drafted the article; Testoni PA and Viale E contributed to critical revision of the article for important intellectual content; Testoni PA and Viale E contributed to final approval of the article.
Correspondence to: Pier Alberto Testoni, MD, Division of Gastroenterology and Gastrointestinal Endoscopy, Vita-Salute San Raffaele University, Scientific Institute San Raffaele, Via Olgettina 60, 20132 Milan, Italy. testoni.pieralberto@hsr.it
Telephone: +39-2-26432756 Fax: +39-2-26435609
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Abstract

AIM: To investigate if high-definition (HD) colonoscope with i-Scan gave a higher detection rate of mucosal lesions *vs* standard white-light instruments.

METHODS: Data were collected from the computerized database of the endoscopy unit of our tertiary referral center. We retrospectively analyzed 1101 consecutive colonoscopies that were performed over 1 year with standard white-light (n = 849) or HD+ with i-Scan (n = 252) instruments by four endoscopists, in an outpatient setting. Colonoscopy records included patients' main details and family history for colorectal cancer, indication for colonoscopy (screening, diagnostic or surveillance), type of instrument used (standard white-light or HD+ plus i-Scan), name of endoscopist and bowel preparation. Records for each procedure included whether the cecum was reached or not and the reason for failure, complications during or immediately after the procedure, and number, size, location and characteristics of the lesions. Polyps or protruding lesions were defined as sessile or pedunculated, and nonprotruding lesions were defined according to Paris classification. For each lesion, histological diagnosis was recorded.

RESULTS: Eight hundred and forty-nine colonoscopies were carried with the standard white-light video colonoscope and 252 with the HD+ plus i-Scan video colonoscope. The four endoscopists did 264, 300, 276 and 261 procedures, respectively; 21.6%, 24.0%, 21.7% and 24.1% of them with the HD+ plus i-Scan technique. There were no significant differences between the four endoscopists in either the number of procedures done or the proportions of each imaging technique used. Both techniques detected one or more mucosal lesions in 522/1101 procedures (47.4%). The overall number of lesions recognized was 1266; 645 in the right colon and 621 in the left. A significantly higher number of colonoscopies recognized lesions in the HD+ plus i-Scan mode (171/252 = 67.9%) than with the standard white-light technique (408/849 = 48.1%) (P < 0.0001). HD+ with i-Scan colonoscopies identified more lesions than standard white-light imaging (459/252 and 807/849, P < 0.0001), in the right or left colon (mean ± SD, 1.62 ± 1.36 *vs* 1.33 ± 0.73, P < 0.003 and 1.55 ± 0.98 *vs* 1.17 ± 0.93, P = 0.033), more lesions < 10 mm (P < 0.0001) or nonprotruding (P < 0.022), and flat polyps (P = 0.04). The cumulative mean number of lesions per procedure detected by the four endoscopists was significantly higher with HD+ with i-Scan than with standard white-light imaging (1.82 ± 2.89 *vs* 0.95 ± 1.35, P < 0.0001).

CONCLUSION: HD imaging with i-Scan during the withdrawal phase of colonoscopy significantly increased the detection of colonic mucosal lesions, particularly small and nonprotruding polyps.

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Key words: Colonoscopy; High-definition+ with i-Scan colonoscopy; White-light colonoscopy; Colonic polyps;

Nonprotruding lesions; Adenoma detection rate; Withdrawal time; Surface enhancement; Contrast enhancement; Tone enhancement

Peer reviewer: Dr. Jeff Butterworth, MB, FRCP, Department of Gastroenterology, Shrewsbury and Telford Hospitals NHS Trust, 102 The Mount, Shrewsbury SY3 8PH, United Kingdom

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INTRODUCTION

Screening colonoscopy is widely considered the gold standard for detection of colonic neoplasia and adenomatous lesions[1]; however, there are several reports of failure to detect small and flat neoplastic lesions[1-10], meaning that in these cases, colonoscopy does not provide adequate protection against colorectal cancer. This inadequacy results in up to 6% of new or missed cancer 3 years after colonoscopy[11,12]. In a recent study, colonoscopy in the preceding 10 years was associated with an overall 77% lower risk for colorectal cancer and approximately 50% lower risk for right-sided cancer[13].

Major factors affecting this polyp miss rate are the presence of blind segments in the colon, poor colon cleaning, and the fact that standard white light may be unable to recognize some small or flat lesions, which are particularly frequent in the right colon. The operator's experience and a longer withdrawal time, permitting closer observation, can only partly overcome these limitations. Even experienced endoscopists may miss up to 6% of adenomas larger than 1 cm and 30% of all adenomas[2,14,15].

Endoscopes have now been designed to improve mucosal visualization, with a wide angle of view and high-resolution, high-definition imaging. Despite these technical improvements, however, there is still debate about the value of high-definition colonoscopy in clinical practice. Out of 11 studies published so far evaluating the capacity of high-definition imaging to improve the lesion detection rate during colonoscopy, five have concluded that it gave no significant advantage over standard white-light colonoscopy[16-26]. A recent meta-analysis evaluating five studies involving 4422 patients and comparing high-definition *vs* standard white-light colonoscopy showed that there were marginal differences between the two imaging technologies for detection of colonic polyps and no advantages of high-definition in the detection of high-risk adenomas[27]. The introduction of instantaneous non-white-light imaging that mimics chromoendoscopy (Narrow-band, Olympus Ltd. and FICE, Fujinon Ltd.) makes it possible to enhance contrast and potentially to improve the detection of mucosal lesions; these filter techniques significantly raised the polyp detection rate in all but three of 13 studies to date[16,28-39]. However, two meta-analyses gave conflicting results[38,40].

A newly developed post-processing filter technology, the i-Scan (Pentax Ltd., Tokyo, Japan), combined and integrated into a high-definition processor (EPKi) that generates images above the high-definition television standard (HD+ resolution), highlights the mucosal surface and architecture by surface enhancement (SE), contrast enhancement (CE), and tone enhancement (TE) modes. So far, in all reports but one, retrospective, it permitted significantly better recognition and characterization of the mucosal lesions during colonoscopy[41-45]. In one recent study, narrow-band imaging and i-Scan significantly improved the polyp detection rate and showed similar efficacy[46].

However, most of the studies using these new post-processing filter techniques are based on prospective, controlled clinical trials in a limited number of patients, in which endoscopists are likely to do the colonoscopy more diligently than in routine practice, with adequate bowel preparation, so it is not clear whether the better polyp detection rates reported can be maintained in routine practice.

The aim of the present study was therefore to determine whether the routine use of colonoscopes equipped with high-definition combined with i-Scan technology (HD+ plus i-Scan) gave a higher rate of detection of overall mucosal lesions, particularly of flat adenomas, than standard white-light video colonoscopes, in a consecutive series of patients undergoing screening, diagnostic or surveillance colonoscopy by different endoscopists with similar expertise, in an outpatient clinical practice setting.

MATERIALS AND METHODS

Data for the study were collected from the computerized database of the endoscopy unit of our tertiary referral center. Colonoscopy records included patients' main details and family history for colorectal cancer, indication for colonoscopy (screening, diagnostic or surveillance), type of instrument used (standard white-light or HD+ plus i-Scan), name of endoscopist, and bowel preparation, defined on the basis of a modified Ottawa scale[47].

Records for each procedure included whether the cecum was reached or not and the reason for failure (inadequate cleaning, strictures, and pain during the procedure), complications during or immediately after the procedure, and number, location and characteristics of the lesions. Polyps or protruding lesions were defined as sessile (Ⅰs) or pedunculated (Ⅰp), and nonprotruding lesions as elevated (Ⅱa), flat (Ⅱb), and depressed (Ⅱc), according to Paris classification[48]. For each lesion, histological diagnosis was recorded. Size and location of the lesions were classified as follows: 0-5 mm, 6-10 mm, 11-15 mm, 16-20 mm, 21-30 mm, > 30 mm; right and left colon. Withdrawal time was recorded for all screening colonoscopies, being

these procedures the object of other studies. Images of each lesion were stored in the database. For each patient, pO_2, heart rate, and blood pressure were measured and recorded before, during and at the end of the procedure.

Data collection

Over a 1-year period, all consecutive screening, diagnostic and surveillance colonoscopies in outpatients done by four expert endoscopists, each of whom had done 200-400 colonoscopies/year for at least 15 years and at least 50 procedures with HD+ plus i-Scan definition equipped instruments were evaluated. The four endoscopists used the two endoscopy techniques in a random fashion, depending of the availability of the instruments. Colonoscopies in subjects younger than 18 years, with genetic-associated colon cancer risk conditions, acute gastrointestinal bleeding, or inflammatory bowel disease were excluded. Procedures with insufficient bowel cleansing, patients in whom residual stool could not be removed by endoluminal washing and suctioning, and patients in whom the cecum was not reached were also excluded.

All patients gave informed consent for the procedures, diagnostic or therapeutic, and for data management for scientific purposes. The retrospective, observational study was approved by the institutional ethics committee.

Examination technique

The bowel was prepared in all cases with polyethylene glycol: 4 L SELG (Promefarm S.r.l, Milan, Italy) or 3 L Moviprep (Norgine GmbH, Marburg, Germany), divided into two parts, were taken the day before the procedure. All patients received conscious sedation with midazolam (Ipnovel, Roche SPA, Basel, Switzerland) and fentanyl (Fentanest, Pfizer, New York, United States) or deep sedation with propofol (Diprivan, AstraZeneca, Zug, Switzerland); 20 mg Butylscopolamin (Buscopan, Boehringer Ingelheim Pharma GmbH, Ingelheim, Germany) were administered if necessary, unless contraindicated.

Standard white-light video colonoscopy was carried out with Pentax colonoscopes EC-3870FZK, EC 3885F, EC 3885L (Pentax Ltd., Tokyo, Japan) and an EPM 3500 or EPK 1000 processor. The colon was inspected during withdrawal of the instrument and lesions were identified and characterized with light imaging only. Magnification was not possible with these endoscopes.

HD+ plus i-Scan video colonoscopy was carried out with Pentax colonoscopes EC-3890FI and EC 3870FZK, using the EPKi processor. The i-Scan technology is a digital contrast method using a light filter that uses different software algorithms with real-time image mapping embedded in the EPKi processor. It enhances mucosal imaging by activating three distinct functions-one for SE mode, the second for CE mode, and the third for TE mode. For SE and CE, there are three enhancement levels (low, medium and high); TE mode can be specifically tailored for the esophagus, stomach, or colon. SE mode enhances the structure through recognition of the edges; compared to normal images, SE images do not differ in brightness and differ little in color, but allow easier recognition of minute glandular structures, which makes it simpler to check changes on the basis of structural differences. With CE mode, areas with lower luminance intensity than surrounding pixels are identified on the basis of pixel-wise luminance intensity data. Processing images with CE does not change the image brightness but enhances minute irregularities and depressed areas of the mucosal surface with a slight bluish-white stain. With TE mode, the RGB components of an ordinary endoscope image are broken down into their parts, and each one is then converted independently along the tone curve, followed by resynthesis of the three components to yield a reconstructed image[43].

The three modes are arranged in series, so two or more can be applied at one time. The modes of enhancement and their levels can be switched on a real-time basis, permitting efficient endoscopic observation.

In all cases, colonoscopy was done using the SE (low) + CE (low) modes; the TE mode for the colon was routinely activated during withdrawal of the instrument once the cecum had been reached, so the whole retrieval phase of the procedure was done using the i-Scan technique with TE mode set for the colon.

Statistical analysis

Data were analyzed using SPSS version 17.0 software (Chicago, IL, United States). Continuous data were described by mean and standard deviation or compared with the Mann-Whitney test. Statistical differences in categorical variables were analyzed using two-sided Fisher's exact tests or χ^2 tests, as appropriate. All differences were considered significant at two-sided P value < 0.05.

RESULTS

A total of 1101 colonoscopy records with images obtained by the four endoscopists were eligible for the study: 849 with the standard white-light video colonoscope and 252 with the HD+ plus i-Scan video colonoscope. The four endoscopists did 264, 300, 276 and 261 procedures, respectively; 21.6%, 24.0%, 21.7% and 24.1% of them with the HD+ plus i-Scan technique. The number of colonoscopies carried out for screening, diagnosis, and surveillance with standard white-light and HD+ plus i-Scan technology by the four endoscopists are reported in Table 1. There were no significant differences between the four endoscopists in either the number of procedures done or the proportions of each imaging technique used.

Both techniques detected one or more mucosal lesions in 522/1101 procedures (47.4%). The overall number of lesions recognized was 1266: 645 in the right colon and 621 in the left. A significantly higher number of colonoscopies recognized lesions in the HD+ plus i-Scan mode (171/252 = 67.9%) than with the standard white-light technique (408/849 = 48.1%) ($P < 0.0001$). The number of mucosal lesions recognized by the two imaging tech-

Table 1 Colonoscopies carried out for screening, diagnosis and surveillance *n* (%)

Indications	HD+ with i-Scan	Standard white light	Total
Screening	69 (23.9)	219 (76.1)	288
Diagnosis	156 (22.1)	552 (77.9)	708
Follow-up	27 (25.7)	78 (74.3)	105
Total	252	849	1101

HD: High-definition.

Table 2 High-definition+ with i-Scan and standard white-light colonoscopy detection rates of mucosal lesions

Indications	HD+ with i-Scan[1]	Standard white light[1]	*P* value
Screening	179/69 (2.59)	207/219 (0.94)	< 0.0001
Diagnosis	203/156 (1.3)	524/552 (0.94)	0.0105
Follow-up	77/27 (2.8)	76/78 (0.97)	< 0.0001
Total	459/252 (1.82)	807/849 (0.95)	< 0.0001

[1]Number of lesion/procedure (mean). HD: High-definition.

Table 3 Number and size of protruding and nonprotruding lesions found with high-definition+ with i-Scan and standard white-light colonoscopy

	HD+ with i-Scan	Standard white light	Total	*P* value
0-10 mm				< 0.0001
Protruding	341	636	977	
Nonprotruding	43	31	74	
11-20 mm				0.83
Protruding	30	67	97	
Nonprotruding	12	23	35	
21-30 mm				0.36
Protruding	9	8	17	
Nonprotruding	12	21	33	
> 30 mm				
Protruding	9	12	21	0.46
Nonprotruding	3	9	12	

HD: High-definition.

niques and the mean numbers detected by each procedure were significantly higher for HD+ plus i-Scan than with standard white light, for screening, diagnostic, and surveillance colonoscopies (Table 2). In both the right and left colon, HD+ plus i-Scan colonscopy recognized a larger mean number of lesions than standard white light (mean ± SD 1.62 ± 1.36 *vs* 1.33 ± 0.73, $P < 0.003$ and 1.55 ± 0.98 *vs* 1.17 ± 0.93, $P = 0.033$).

Overall, 154 nonprotruding lesions were identified and removed: 70 with the HD+ plus i-Scan mode (27.8%) and 84 with the standard white-light technique (9.9%). The HD+ plus i-Scan mode recognized a significantly higher number of nonprotruding lesions than the standard white-light technique ($P = 0.04$) (Figures 1 and 2).

The overall number and size of the lesions, protruding or nonprotruding, found with HD+ plus i-Scan and standard white light are shown in Table 3. The HD+ plus i-Scan technique identified a significantly larger number of lesions smaller than 10 mm, either protruding or nonprotruding, than standard white light ($P < 0.0001$); the difference was not significant for lesions measuring 11-20 mm, 21-30 mm, and > 30 mm. Colonoscopies performed with HD+ with i-Scan technique also identified a significantly larger number of overall lesions and nonprotruding lesions smaller than 10 mm than did standard white light ($P < 0.0001$ and $P < 0.022$, respectively), while the difference was not different for larger lesions, either protruding or nonprotruding. The differences were not significant considering screening, diagnostic, and surveillance colonoscopies.

Among the 154 nonprotruding lesions, histological report was available for 133 lesions, because in 21 cases, resected specimens were missed during colonoscopy (Table 4). Adenoma detection rate was significantly higher with HD+ plus i-Scan mode than with standard white light only for lesions smaller than 10 mm (32/35 *vs* 19/27, $P = 0.05$), while the difference was not significant for larger adenomas.

The number of procedures managed by the four endoscopists and the distribution of HD+ plus i-Scan and standard white-light colonoscopies, with the mean numbers of lesions found by each one. The lesion detection rates were very similar for all four. The cumulative mean number of lesions per procedure detected with the two techniques was significantly higher with the HD+ plus i-Scan than with standard white-light imaging (mean ± SD, 1.82 ± 2.89 *vs* 0.95 ± 1.35, $P < 0.0001$). In fact, each of the four endoscopists identified twice as many lesions with the HD+ plus i-Scan as with standard white-light imaging.

The overall withdrawal time, reported only for screening colonoscopies, did not significantly differ between procedures performed with the HD+ plus i-Scan and standard white light (8.4 ± 1.2 min *vs* 8.3 ± 1.4 min, respectively) (Table 5).

DISCUSSION

To date, only one study has evaluated the impact of the routine use of i-Scan with TE mode and HD+ imaging in the detection of mucosal lesion during the withdrawal phase of colonoscopy, compared to standard white-light imaging, in a large series of patients in clinical practice[45]. The study was retrospective and did not improve adenoma detection rate in a population with mixed risk for colorectal cancer.

In our retrospective study, with the HD+ plus i-Scan imaging routinely activated during the withdrawal phase of colonoscopy, once the cecum had been reached, a significantly larger number of examinations identified some mucosal lesion and adenomas, either protruding or flat, and there were also significant improvements in the overall detection rate of lesions and the mean number of lesions recognized for each colonoscopy, compared with standard white-light imaging. The rate was most markedly higher for lesions not bigger than 10 mm and nonprotruding ones. Although the rates of detection of

Table 4 Histological report of nonprotruding lesions

	0-10 mm		11-20 mm		21-30 mm		> 30 mm		Total
	HD + with i-Scan	Standard white light	HD + with i-Scan	Standard white light	HD + with i-Scan	Standard white light	HD + with i-Scan	Standard white light	
Missing	8	4	0	3	0	3	0	3	21
Hyperplastic	3	8	3	2	3	0	0	0	19
Serrated	29	16	6	18	9	18	0	0	96
LGIN	3	3	0	0	0	0	3	6	15
Adenocarcinoma	0	0	3	0	0	0	0	0	3
Total	43	31	12	23	12	21	3	9	154

HD: High-definition; LGIN: Low grade intraepithelial neoplasia.

Table 5 Procedures performed by the four endoscopists using the two techniques

Operator	Procedures	HD+ with i-Scan	Standard white light	Lesions	No. of lesions (mean number of lesions/procedure)		*P* value
					HD+ with i-Scan	Standard white light	
1	264	57	207	330	117 (2.05)	213 (1.02)	< 0.0001
2	300	72	228	375	132 (1.83)	243 (1.07)	0.089
3	276	60	216	294	114 (1.9)	180 (0.83)	< 0.0001
4	261	63	198	267	96 (1.52)	171 (0.86)	0.71
Total	1101	252	849	1266	459 (1.82)	807 (0.95)	< 0.0001

HD: High-definition.

lesions larger than 10 mm did not differ with the two imaging techniques, protruding and nonprotruding lesions smaller than 10 mm were recognized significantly more frequently using the HD+ plus i-Scan technology. In particular, HD+ plus i-Scan technology identified flat polyps smaller than 10 mm three times more than the white-light technique.

The cumulative mean number of lesions per colonoscopy recognized by the four colonoscopists was significantly higher with HD+ plus i-Scan than with standard white-light imaging, while the withdrawal time, when recorded, did not differ between the two techniques.

Only two studies published so far have assessed the combined use of HD+ plus i-Scan for colonoscopy; they have reported similar results in favor of this technique but they were obtained in a prospective trial setting and in a smaller number of selected patients[42,44].

Identifying more polyps by colonoscopy in clinical practice, including small (< 10 mm) and flat ones, may have an important impact for colorectal cancer prevention. The polyp miss rate is probably the main factor accounting for a persistent risk of colorectal cancer reported in 10%-24% of cases after screening colonoscopy[49].

A systematic review of six tandem colonoscopy studies using standard white-light imaging showed an overall polyp miss rate of 22%. The rate rose with smaller lesions, ranging from 2.1% for lesions bigger than 10 mm, to 13% for those between 5 and 10 mm, and up to 26% for those smaller than 5 mm[8]. A prospective multicenter study of back-to-back colonoscopies with white-light imaging reported 9% and 27% miss rates for adenomas > 5 mm and < 5 mm, respectively, and 11% for advanced adenomas[9]. This means that small and flat mucosal lesions, mostly in the right colon, are the ones that may frequently be missed during colonoscopy.

A limited number of studies have compared the efficacy of HD+ colonoscopy with standard white-light colonoscopy, and the findings are far from clear: four of the nine studies concluded that high-definition imaging gave no benefit compared to standard resolution[17,18,21,24]. The addition of electronic filters, such NBI and FICE, to the high-definition imaging did improve the polyp detection rate for small/flat lesions but some results were still disappointing for this end-point[16,30].

Even though there is a general belief that detecting and removing small lesions (1-5 mm) in the colon may not have any significant clinical impact, a number of studies have found that small lesions, mainly flat ones, may have unfavorable histology. One reported that small depressed colorectal lesions had up to a 40% chance of submucosal invasion[49]; two found that 3.9% and 16% of adenomas between 6 and 10 mm had high-grade dysplasia[50,51], and 0.5% of adenomas measuring 6-9 mm were actually cancer[51]. These data might explain the reported occurrence of colorectal cancer after negative screening colonoscopy and support the need for detecting and removing all protruding lesions of the colon, regardless of the size, and selecting the most appropriate techniques to ensure maximum recognition of lesions at colonoscopy.

HD+ plus i-Scan can also differentiate diminutive adenomas and hyperplastic polyps[52], and a recent study using a Markov simulation model suggested that a resect and discard strategy for very small polyps might improve the cost-effectiveness of colorectal cancer screening[53].

A potential limitation of the present study was its retrospective nature. However, data used for analysis, in-

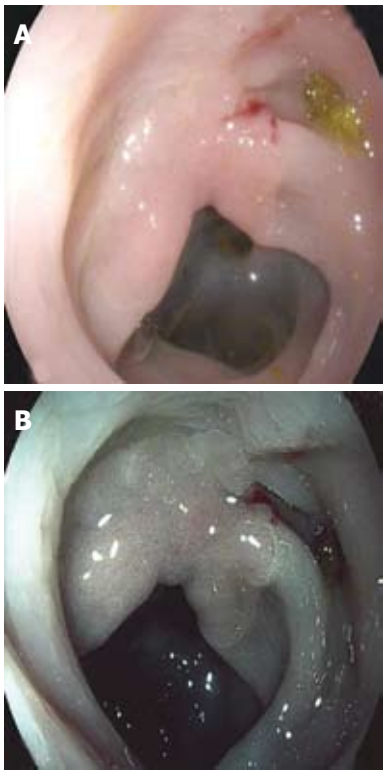


Figure 1 Flat lesion Ⅱb + Ⅱa on left colon examined by high-definition-white light and visualized with i-Scan. A: Flat lesion Ⅱb + Ⅱa of 25 mm × 25 mm on left colon examined by high-definition white light; B: Same lesion visualized with i-Scan.

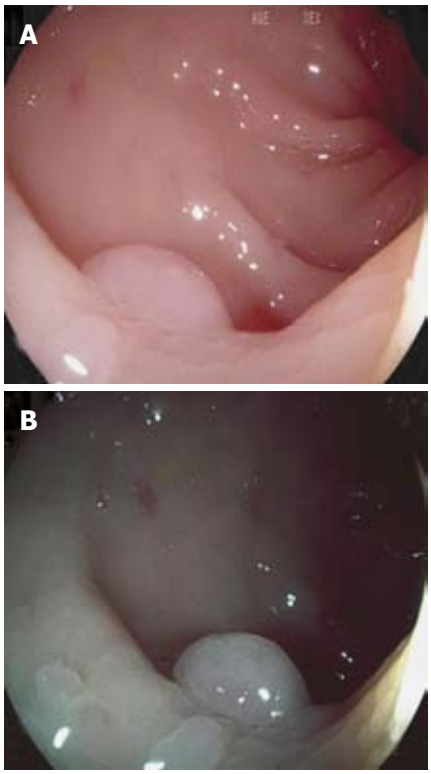


Figure 2 Flat lesion 0-Ⅱa visualized with high-definition white light and surface enhancement and visualized with i-Scan and digital chromoendoscopy. A: Flat lesion 0-Ⅱa visualized with high-definition white light and surface enhancement; B: Same lesion visualized with i-Scan and digital chromoendoscopy.

cluding the adequacy of bowel preparation, were detailed and were collected prospectively for each procedure and stored in a database. Only procedures that included all the data required for the study were considered. As with all nonrandomized trials, potential confounding variables cannot be entirely excluded; however, we examined a large number of colonoscopies and statistical analysis found highly significant differences. Although colonoscopies carried out for different purposes (screening, diagnostic and surveillance) may represent different settings, the differences reported from overall results were also confirmed in the three settings. On the other hand, the retrospective design has the advantage of providing information on the true yield of HD+ plus i-Scan imaging for detecting polyps during colonoscopy in current clinical practice. Prospective trials evaluating new imaging systems could allow the endoscopist to be more attentive during the procedures outside routine practice and very likely give greater accuracy for polyp detection, especially for flat and small lesions, but the good results are not necessarily directly transferable into routine clinical practice.

The lack of documentation of withdrawal time for all colonoscopies is another potential limitation of a retrospective study, compared with prospective ones, because withdrawal time plays an important role in adenoma detection, although here too data are conflicting. In this retrospective evaluation, we were able to assess reliably the withdrawal times only for screening colonoscopies without therapeutic interventions: withdrawal time was comparable by using the two imaging techniques. Besides the imaging technology, probably the endoscopist's technique and experience is perhaps more important than other factors, including withdrawal time, in detecting polyps by colonoscopy[54-56]. The endoscopists in this study were experts, with many colonoscopies behind them and on their current schedules, and adequate experience with HD+ plus i-Scan imaging in the year leading up to the study. In addition, we compared the numbers of colonic lesions recognized by the same endoscopist using the two techniques, thus applying similar expertise and technique, in a similar clinical setting, and found that the four endoscopists using HD+ plus i-Scan imaging detected cumulatively more lesions. Only one other study comparing the diagnostic yield for colonic polyps using standard white-light and HD+ colonoscopy followed a retrospective design, with an adequate number of unselected patients undergoing colonoscopy in routine practice. The findings confirmed the greater accuracy for detecting polyps of HD imaging compared with white light (42.2% *vs* 37.8%)[20]. In our hands, 67.8% and 27.8% of colonoscopies with HD+ plus i-Scan recognized some mucosal lesions and flat small polyps (< 10 mm), respectively, compared to 48.1% and 9.9% for standard white-light imaging. HD+ plus i-Scan thus gave an approximately 30% higher diagnostic yield for mucosal lesions of the colon and increased by three times the diagnostic accuracy for flat polyps smaller than 10 mm.

In conclusion, this retrospective study on a large se-

ries of consecutive outpatients undergoing colonoscopy in different settings by four expert endoscopists showed that the routine addition of i-Scan to HD imaging during the entire withdrawal phase of colonoscopy, once the cecum had been reached, significantly increased the diagnostic yield for detection of mucosal lesions of the colon, particularly small and nonprotruding ones, without affecting the withdrawal time. In colon cancer screening, the routine use of HD+ plus i-Scan can recognize more mucosal lesions without the need to prolong the withdrawal time to allow for closer inspection, as suggested in other studies, and could probably enable less-skilled endoscopists to achieve performances comparable to those of experienced ones in detecting colonic polyps.

COMMENTS

Background

Screening colonoscopy is widely considered the gold standard for detection of colonic neoplasia and adenomatous lesions, however, there are several reports of failure to detect small and flat neoplastic lesions, meaning that in these cases, colonoscopy does not provide adequate protection against colorectal cancer. Besides the operator's experience, withdrawal time, quality of colon cleansing, presence of blind segments in the colon, and quality of imaging provided by endoscopes play an important role in lesion detection. Standard white-light imaging may be unable to recognize some small or flat lesions, which are particularly frequent in the right colon, and it may affect the polyp miss rate during routine colonoscopy. High-definition (HD) imaging and filter technologies have been applied to colonoscopies to improve detection of lesions, but results are conflicting.

Research frontiers

Endoscopes have now been designed to improve mucosal visualization, with a wide angle of view, filter-aided techniques that can enhance characterization of mucosal morphology and surface architecture, and high-resolution/high-definition imaging that can improve endoscopic recognition of mucosal lesions. In this study, the authors demonstrated that the routine use of HD+ plus i-Scan recognized more mucosal lesions without the need to prolong the withdrawal time to allow closer inspection.

Innovations and breakthroughs

Recent studies have analyzed the capacity of high-definition imaging to improve the lesion detection rate during colonoscopy with conflicting results. The value of high-definition colonoscopy in clinical practice is still debated. In this study, the authors showed that the routine addition of i-Scan to HD+ imaging during the entire withdrawal phase of the colonoscopy significantly increased the diagnostic yield for detection of mucosal lesions of the colon, particularly small and nonprotruding ones, without affecting the withdrawal time, and could probably enable less-skilled endoscopists to achieve performances comparable to those of experienced ones in detecting mucosal lesions.

Applications

This study may encourage the utilization of advanced imaging technologies to reduce polyp miss rate and improve colonoscopy performance in the prevention of colorectal cancer.

Terminology

The i-Scan technology is a digital contrast method employing a light filter that uses different software algorithms with real-time image mapping embedded in the Pentax EPKi processor. i-Scan enhances mucosal imaging by activating three distinct functions: one for surface enhancement (SE), the second for contrast enhancement (CE), and the third for tone enhancement (TE), allowing a better recognition and characterization of the mucosal lesions during colonoscopy. SE mode enhances the structure through recognition of the edges, compared to normal images, and allows easier recognition of minute glandular structures which makes it simpler to identify changes on the basis of structural differences. CE mode enhances minute irregularities and depressed areas of the mucosal surface with a slight bluish-white stain. In TE mode, the RGB components of an ordinary endoscope image are broken down into their parts, and each one is then converted independently along the tone curve, followed by resynthesis of the three components to yield a reconstructed image.

Peer review

The authors examined the role of HD+ i-Scan *vs* white-light colonoscopy on polyp detection rates. The research is a significant addition to the literature on the use of contrast technology in improving the quality of colonoscopy in detecting polyps. The results of the study will encourage those regularly involved in performing colonoscopy to consider a lower threshold in utilizing these techniques to improve polyp detection rates. The research novelty is in the fact that the study was conducted in a real clinical practice environment and could be considered to have greater clinical applicability.

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BRIEF ARTICLE

Graft-versus-host disease after liver transplantation: A comprehensive literature review

Sami Akbulut, Mehmet Yilmaz, Sezai Yilmaz

Sami Akbulut, Mehmet Yilmaz, Sezai Yilmaz, Liver Transplant Institute, Inonu University Faculty of Medicine, 44280 Malatya, Turkey
Author contributions: Akbulut S, Yilmaz M and Yilmaz S contributed to writing the article and reviewing the literature, as well as undertaking a comprehensive literature search; and Akbulut S performed the statistical analyses.
Correspondence to: Dr. Sami Akbulut, MD, Department of Surgery, Liver Transplantation Institute, Inonu University Faculty of Medicine, 44280 Malatya,
Turkey. akbulutsami@gmail.com
Telephone: +90-422-3410660 Fax: +90-422-3410036
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Abstract

AIM: To determine the factors affecting mortality in patients who developed graft-versus-host disease (GvHD) after liver transplantation (LT).

METHODS: We performed a review of studies of GvHD following LT published in the English literature and accessed the PubMed, Medline, EBSCO, EMBASE, and Google Scholar databases. Using relevant search phrases, 88 articles were identified. Of these, 61 articles containing most of the study parameters were considered eligible for the study. Risk factors were first examined using a univariate Kaplan-Meier model, and variables with a significant association ($P < 0.05$) were then subjected to multivariate analyses using a Cox proportional-hazards model.

RESULTS: The 61 articles reported 87 patients, 58 male and 29 female, mean age, 40.4 ± 15.5 years (range: 8 mo to 74 years), who met the inclusion criteria for the present study. Deaths occurred in 59 (67.8%) patients, whereas 28 (32.2%) survived after a mean follow-up period of 280.8 ± 316.2 d (range: 27-2285 d). Among the most frequent symptoms were rash (94.2%), fever (66.6%), diarrhea (54%), and pancytopenia (54%). The average time period between LT and first symptom onset was 60.6 ± 190.1 d (range: 2-1865 d). The Kaplan-Meier analysis revealed that pancytopenia (42.8% *vs* 59.3%, $P = 0.03$), diarrhea (39.2% *vs* 61.0%, $P = 0.04$), age difference between the recipient and the donor (14.6 ± 3.1 years *vs* 22.6 ± 2.7 years, $P < 0.0001$), and time from first symptom occurrence to diagnosis or treatment (13.3 ± 2.6 mo *vs* 15.0 ± 2.3 mo, $P < 0.0001$) were significant factors affecting mortality, whereas age, sex, presence of rash and fever, use of immunosuppressive agents, acute rejection before GvHD, etiological causes, time of onset, and donor type were not associated with mortality risk. The Cox proportional-hazards model, determined that an age difference between the recipient and donor was an independent risk factor ($P = 0.03$; hazard ratio, 7.395, 95% confidence interval, 1.2-46.7).

CONCLUSION: This study showed that an age difference between the recipient and donor is an independent risk factor for mortality in patients who develop GvHD after LT.

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Key words: Liver transplantation; Graft-versus-host disease; Immunosuppression; Rash; Pancytopenia; Diarrhea; Chimerism; Age factors

Peer reviewer: Rui Tato Marinho, Professor, Department of Gastroenterology and Hepatology, Hospital Santa Maria, Rua Prof. Aires de Sousa, 1 r/c A, 1600-590 Lisboa, Portugal

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INTRODUCTION

Graft-versus-host disease (GvHD) results from the reac-

tion of donor immunocompetent cells against tissues of an immunosuppressed host[1-5]. GvHD is a well-known complication in patients who undergo allogeneic bone-marrow transplantation. However, few reports of GvHD after solid-organ transplantation include liver transplantation (LT)[6-9]. The reported incidence of this complication varies from 0.1% to 2%, with a mortality rate of > 75%; GvHD usually occurs between the second and sixth week after LT[5,10,11]. The clinical manifestations of GvHD following LT include fever, rash, diarrhea, and hematocytopenia, but the basic function of the transplanted liver is not affected[2,9,12,13]. The diagnosis of GvHD following LT can be difficult, as many of the clinical signs can be caused by drug reactions or viral infections including cytomegalovirus (CMV)[9]. Although a sizable number of modalities have been used to manage this disease, the most effective combination has not been determined.

MATERIALS AND METHODS

The primary purpose of this study was to examine the existing literature on GvHD following LT. Thus, we conducted a thorough literature search regarding GvHD developing after LT using the PubMed, Medline, EBSCO, EMBASE, and Google Scholar databases from November 2011 to March 1988, when Burdick *et al*[14] presented the first study on GvHD following LT. The keywords we used for the search were "graft-versus-host disease," "graft-versus-host disease after liver transplantation," "graft-versus-host disease following liver transplantation," and "graft-versus-host disease and solid-organ transplantation." The reference lists of all articles introduced as reviews were checked to attain a wider search range. The search identified 88 article titles. More detailed information was requested through contact with the corresponding authors and/or the related journal editors for studies in which insufficient data were provided or in which full texts could not be accessed. Twenty-seven full-text articles were excluded from the study because the authors could not be reached, a case presentation was duplicated, or only a literature review was provided that did not include sufficient information for comparison with other studies. A total of 61 articles containing most of the parameters mentioned below were considered eligible for the study. One of the two cases presented by Schuchmann *et al*[15] was excluded because it was presented in another study. The study by Knox *et al*[16] was excluded because only pulmonary GvHD developed following LT. In the 61 studies for which full texts could be accessed, the following data were evaluated: age, sex, donor age, age difference between the recipient and the donor, blood group compatibility (identical or not), donor type (living/cadaveric), use of primary immunosuppressive medications (tacrolimus, cyclosporine, or azathiopurine), primary hepatic disease, time of onset (postoperative day), first manifestations (rash, fever, pancytopenia, thrombocytopenia, leukopenia, and diarrhea), time interval elapsed between the first manifestation and the diagnosis and/or treatment (d), re-transplantation, mortality, and follow-up. The aim of this literature search was to identify factors affecting the occurrence of mortality in post-transplantation GvHD. Thus, the patients were divided into a mortality group (n = 59) and a survival group (n = 28). Accordingly, symptoms such as fever, rash, and diarrhea were collected under the title of "first symptoms" after ruling out other possible causes. Similarly, pancytopenia, thrombocytopenia, or leukopenia that developed before the confirmation of the GvHD diagnosis were all collected under the title of "pancytopenia." Symptoms or hematological disorders developing after commencement of treatment were left out of the former classifications. The time period between development of the first symptom associated with the disease and the transplantation was termed "time of onset."

Statistical analysis

SPSS version 13.0 (SPSS, Inc., Chicago, IL, United States) was used for the statistical analysis. Data are presented as mean ± SD for continuous variables and as frequencies for categorical variables. The statistical significance of differences between groups was examined using Pearson's χ^2 test for categorical variables and the Student t-test for continuous variables. Risk factors for outcomes were first examined using a univariate Kaplan-Meier model, and variables with a significant association ($P < 0.05$) were then subjected to multivariate analyses using a Cox proportional-hazards model. All statistical tests were two-sided with a significance level of 0.05.

RESULTS

We retrospectively evaluated 61 studies that included 87 patients, 58 male and 29 female, with age range of 8 mo to 74 years (mean, 40.4 ± 15.5 years). There were 59 (67.8%) deaths, while 28 (32.2%) survived at a mean follow-up of 280.8 ± 316.2 d (range: 27-2285 d). In the Kaplan-Meier model, parameters such as pancytopenia (P = 0.03), diarrhea (P = 0.04), age difference between the recipient and the donor ($P < 0.0001$), and the time elapsed between development of the first symptoms and the diagnosis or treatment ($P < 0.0001$) were significant risk factors for mortality. The results of multivariate Cox proportional-hazards model analysis revealed that age difference was an independent and strong risk factor (P = 0.03; hazard ratio, 7.395, 95% confidence interval, 1.2-46.7). Kaplan-Meier mortality curves for patients with and without diarrhea and pancytopenia are presented in Figure 1. Demographic and statistical data for the mortality and survival groups is provided in Tables 1 and 2. The distribution of both groups by time since the first description of GvHD after LT is depicted in Figure 2. We noted that mortality rates peaked in some years and that cases in the survival group pursued a more stable course compared with those in the mortality group.

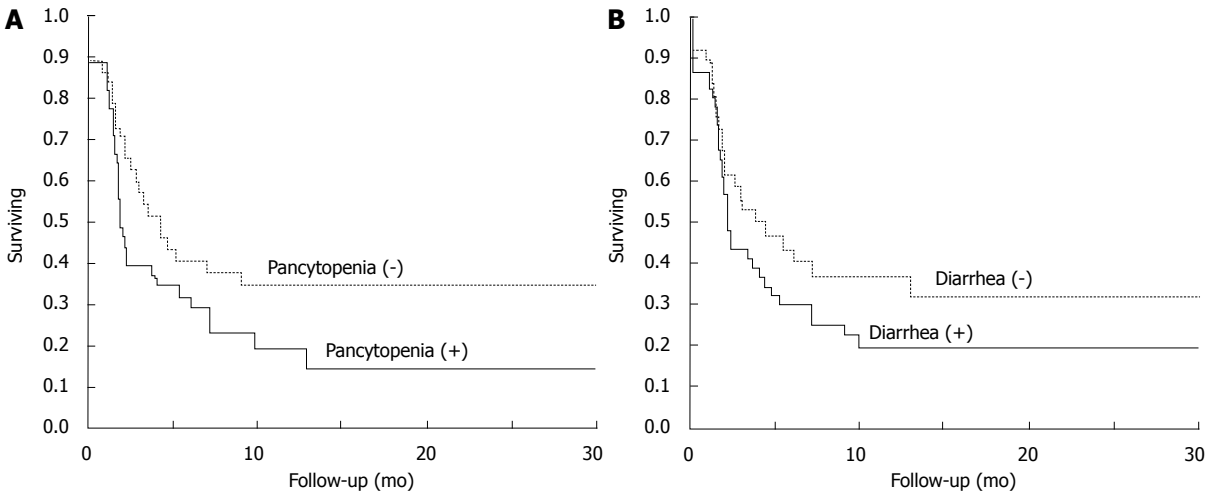


Figure 1 Kaplan-Meier survival curves for patients with and without pancytopenia and diarrhea. A: Pancytopenia; B: Diarrhea.

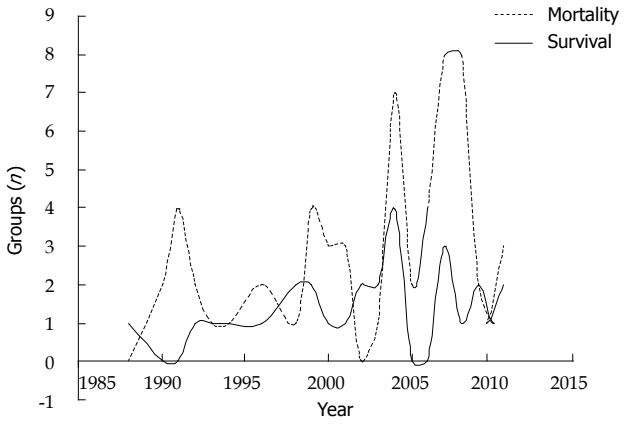


Figure 2 Distribution of survival and mortality groups by time of first description of graft-versus-host disease after liver transplantation.

DISCUSSION

Description, pathogenesis, and incidence of GvHD after liver transplantation

GvHD was first described by Billingham in 1966 as a reaction of the donor's immunocompetent cells against the recipient's cellular antigens[17,18]. The development of GvHD implies the fulfillment of three prerequisites: (1) a source of immunocompetent lymphocytes; (2) histocompatible antigenic differences between donor and host; and (3) inability of the host to reject donor lymphocytes[10,19-21]. This reaction occurs in as many as 80% of patients after bone-marrow transplantation. It has also been infrequently reported after transfusion of blood products or after solid-organ transplantation, such as pancreas-spleen, heart-lung, and liver[6,14,17,22,23]. The development of GvHD after solid-organ transplantation was first defined in 1984 by Starzl *et al*[24] in a patient undergoing a combined pancreas and splenic transplantation operation. GvHD developing after LT was first defined by Burdick *et al*[14] in 1988.

Although the exact mechanisms are still unclear, the three basic prerequisites mentioned above are also applicable to GvHD after LT. An estimated 10^9-10^{10} donor lymphocytes remain in the portal tracts and the parenchyma of a donor liver graft after flushing with cold preservative solution[18,21,25-27]. These T-cells are detectable in the peripheral blood and organs of patients during the first weeks after LT[18,20,25,26,28]. The donor lymphocytes colonize the recipient, recognize the host tissue antigens as foreign, and react against the host tissue. In other words, if the "balance of power" between the donor and recipient immune systems favors the donor, donor lymphocytes may be activated, leading to GvHD.

Although the exact incidence of GvHD following LT remains to be determined, various studies have cited rates of 0.1%-2%[27,29-32]. Yuksekkaya *et al*[11] reported that the incidence of GvHD was as high as 22.2% in patients whose donors were mismatched on at least one human leukocyte antigen (HLA) A and B antigens[11,33]. In our examination of 15 articles, we found that GvHD was evident in only 62 (0.06%) of 9492 patients undergoing LT, which was similar to frequencies reported previously[32-36].

Classification of GvHD after liver transplantation

GvHD has been reported after solid-organ transplantation with humoral and cellular presentations. The humoral type, also known as graft-versus-host hemolysis, is characterized by hemolysis and fever and occurs in patients transplanted with ABO-incompatible or nonidentical grafts. The cellular type of GvHD occurs when immunocompetent donor lymphocytes originating from the transplanted liver undergo activation and clonal expansion, allowing them to mount a destructive cellular immune response against recipient tissues. The response is directed against the major histocompatibility complex and often results in severe multisystem disease with a high mortality rate[19,37-40].

GvHD responses can be classified as acute or chronic, depending on the timing and character of alloimmune activity[11,41,42]. Acute GvHD comprises all manifestations that occur during the first 100 d after transplantation, and chronic GvHD includes all manifestations that oc-

Table 1 Comparison of variables between surviving and dead patients *n* (%)

Characteristics	Surviving (*n* = 28)	Dead (*n* = 59)	Total (*n* = 87)	Univariate analysis	Multivariate analysis
Age, yr	38.7 ± 22.7	40.4 ± 15.5	40.4 ± 15.5	0.1	
Sex				0.8	
Male	20 (71.4)	38 (64.4)	58 (66.6)		
Female	8 (28.6)	21 (35.6)	29 (33.4)		
Rash				0.8	
Present	27 (96.4)	55 (93.2)	82 (94.2)		
Absent	1 (3.6)	4 (6.8)	5 (5.8)		
Fever				0.1	
Present	17 (60.7)	41 (69.5)	58 (66.7)		
Absent	11 (39.3)	18 (30.5)	29 (33.3)		
Pancytopenia				0.03	0.6
Present	12 (42.9)	35 (59.3)	47 (54)		
Absent	16 (57.1)	24 (40.7)	40 (46)		
Diarrhea				0.04	0.1
Present	11 (39.3)	36 (61)	47 (54)		
Absent	17 (60.7)	23 (39)	40 (46)		
Acute rejection before GvHD				0.4	
Yes	7 (25)	8 (13.6)	15 (17.3)		
No	21 (75)	51 (86.4)	72 (82.7)		
Immunsuppressive agent				0.5	
Tacrolimus	15 (53.6)	25 (42.4)	40 (46)		
Cyclosporine	10 (35.7)	20 (33.9)	30 (34.5)		
Azathiopurine	0 (0)	1 (1.7)	1 (1.1)		
Un-noted	3 (10.7)	13 (22)	16 (18.4)		
Re-transplantation				0.4	
Yes	2 (92.9)	2 (3.4)	4 (4.6)		
No	26 (7.1)	57 (96.6)	83 (95.4)		
Time of onset (POD), d	109 ± 64	38 ± 5	60.6 ± 190.11	0.4	
Etiology				0.3	
Donor type				0.2	
Cadaveric	16 (57.1)	16 (27.1)	32 (36.8)		
Living	2 (7.2)	8 (13.6)	10 (11.5)		
Un-noted	10 (35.7)	35 (59.3)	45 (51.7)		
Age difference between recipient and donor, yr	14.6 ± 3.1	22.6 ± 2.7	19.8 ± 13.2	< 0.0001	0.03[1]
Blood group				0.4	
Identical	16 (57.1)	30 (51)	46 (52.9)		
Non-identical	3 (10.7)	3 (5)	6 (6.9)		
Un-noted	9 (32.1)	26 (44)	35 (40.2)		
Time between symptoms and diagnosis or first treatment, mo	13.3 ± 2.6	15.0 ± 2.3	14.3 ± 14.3	< 0.0001	0.1

[1]Hazard ratio: 7.3, 95% confidence interval: 1.2-46.7. GvHD: Graft-versus-host disease; POD: Post-operative day.

cur after 100 d[11,42-44]. However, multiple findings suggest that this may no longer be a suitably useful distinction. Acute GvHD lesions may be found after 100 d, whereas chronic GvHD lesions sometimes appear before 100 d. Acute GvHD histological findings can be found in biopsies performed after day 100, and lichenoid findings can be found in biopsies performed before day 100[42,44]. The number of days after transplant is an insufficient criterion to distinguish acute from chronic GvHD. Good clinical and pathological descriptions are needed. Chronic GvHD can occur as a progression of acute GvHD, as a recurrence following a disease-free interval, or without a history of acute GvHD. Each of these forms accounts for approximately one-third of cases.

Risk factors for development of GvHD

The causes of GvHD following organ transplantation have not been clarified, but several risk factors have been implicated, including close HLA matching between the recipient and donor[18,27,45], blood transfusion prior to transplantation[11], immunosupressive treatment before transplantation[11,27], glucose intolerance[35], rejection before GvHD[4], autoimmune hepatitis[35], alcoholic liver disease[35], hepatocellular carcinoma (HCC)[27,35], re-transplantation[27], a large age discrepancy between donor (younger) and recipient (older)[25], recipient age > 65 years[18,27,45,46], and multiorgan transplantation[5,21,33,35,40]. Only two studies offered an evidence-based risk analysis with regard to the development of GvHD after LT. In a study by Smith *et al*[33], risk factors included recipient age ≥ 65 years, recipient-donor age difference ≥ 40 years, and close matching of the HLA types of the donor to those of the recipient. Chan *et al*[35] documented glucose intolerance, autoimmune hepatitis, alcoholic liver disease, HCC, and various combinations of these but not such parameters as age, sex, ischemia duration, HLA mismatch, or age dif-

Table 2 Distribution of patients according to underlying liver disease

Etiology	Surviving (*n* = 28)	Dead (*n* = 59)	Total (*n* = 87)
ALD	4	7	11
ALD + HCC	1	3	4
ALD + HCV	0	3	3
HBV	1	3	4
HBV + HCC	3	6	9
HBV + HDV	1	0	1
HCV	0	7	7
HCV + HCC	1	0	1
HCC	1	3	4
PBC	1	5	6
PSC	1	3	4
PSC + HCC	0	2	2
Biliary atresia	4	2	6
Hemangioma	0	1	1
Cryptogenic	4	5	9
Acute failure	3	4	7
Autoimmune hepatitis	1	1	2
HAV	1	0	1
Policystic disease	0	1	1
Laennec's cirrhosis	1	0	1
Wilson disease	0	1	1
Langerhans' cell histiocytosis	0	1	1
NRH	0	1	1

ALD: Alcoholic liver disease; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; PBC: Primary biliary chirosis; PSC: Primary sclerosing cholangitis; NRH: Nodular regenerative hyperplasia; HAV: Hepatitis A virus; HBV: Hepatitis B virus.

ferences as risk factors. Chan *et al*[35] argued that most of the risk factors they identified permitted patients to lapse into an immunosuppressive state, suggesting an inclination toward development of the disease before the LT operation. Our literature search showed that the most frequently encountered liver diseases in affected patients were HCC (23%) and alcoholic liver disease (20.7%). The suggestions by Chan *et al*[35] support our findings, but we lack confirming evidence.

Clinical presentation of GvHD

The clinical presentation of GvHD following LT includes skin rash, fever, diarrhea and hematocytopenia[5,47]. Characteristically, the transplanted liver is not a target of GvHD after LT because both graft liver and immunocompetent cells responsible for GvHD are of donor origin[25,28,30]. The most frequently appearing symptoms in our search were rash (94.2%), fever (66.6%), diarrhea (54%), and pancytopenia (54%). Among these symptoms, pancytopenia (P = 0.03) and diarrhea (P = 0.04) were confirmed by univariate analysis to be risk factors affecting mortality. These results indicate that intestinal and bone-marrow involvement may give rise to severe complications.

The clinical symptoms of GvHD usually become apparent between 1 and 8 wk after LT, often after an initial uneventful recovery from surgery and discharge from the hospital[40,48]. Our literature review revealed that the first symptoms appear 60.6 ± 190.1 d (range: 2-1865 d) after the LT operation. Although the time interval was shorter in the non-surviving group, it was not among the risk factors for death (P = 0.4). Despite this result, we believe that the mortality in cases complicated with GvHD within the first month is much higher.

Diagnosis of GvHD

A diagnosis of GvHD after LT is based on the presence of clinical manifestations, a demonstration of chimerism, and histopathological evidence[29,42,47,49]. As the clinical presentation of GvHD is inconsistent, a high degree of suspicion is necessary to pursue a diagnosis. Any or all clinical symptoms mentioned above may be seen during the initial presentation of GvHD. A skin biopsy showing epidermal dyskeratosis with epithelial cell necrosis is highly suggestive but not pathognomonic for GvHD[29,39,40,48]. Chimerism can be established by various methods that examine the presence of donor cells in the recipient's peripheral blood or various tissues[41,50]. These methods include serological HLA typing of peripheral blood, restriction fragment length polymorphism[28,29,51-53], and fluorescent *in situ* hybridization (FISH), which have been used to demonstrate chimerism in recipients with suspected GvHD after LT[28,34,48]. Chimerism at the tissue level has been shown by polymerase chain reaction, short tandem repeat analysis, and FISH techniques in the skin and bone marrow of patients with GvHD after LT[46,48,52,54,55]. Peripheral blood chimerism appears transiently in the majority of patients during the early postoperative period after LT, particularly in the first week, and rapidly declines by the third to fourth week post-transplant[39,45]. For this reason, chimerism may not be evident in the peripheral blood of patients with late-onset GvHD[32,56].

Differential diagnosis and clinical significance

The differential diagnosis of GvHD after LT is frequently delayed because early symptoms are often non-specific. The differential diagnosis consists of (1) drug-induced skin reactions, including toxic epidermal necrolysis and mycophenolate mofetil toxicity; (2) viral exanthemas; (3) infectious enteritis, including CMV infection and *Clostridium difficile* colitis, and (4) organ rejection[13,41,57-59]. Many of the clinical signs of GvHD may also be seen with CMV infection. The presence of CMV in a patient with GvHD may complicate the appropriate diagnosis and delay treatment. A significant association between acute GvHD and CMV after transplant has been documented and may be related to pancytopenia resulting from bone-marrow depletion by attacking donor lymphocytes[9,11,34,39].

A rapid differential diagnosis and early implementation of treatment for GvHD following LT are two factors that affect survival. In contrast, studies showing that early treatment was not effective in the ultimate outcome have also been published[18,40]. Taylor *et al*[40] based their opinions on a literature search. They reported that early implementation of treatment did not produce a statistically significant difference in mortality. We found that the time interval between the appearance of first symptoms

and definitive diagnosis and/or treatment, which ranged from 1 to 65 d, was a statistically significant predictor of death ($P < 0.0001$).

Treatment of GvHD after liver transplantation

The evidence base for selecting the most appropriate therapy for established GvHD after LT is very limited; thus, treatment is largely empirical, although the extensive literature on managing acute GvHD after stem cell transplantation provides guidance[21,25,27,40]. A number of treatment modalities have been proposed based on the known pathophysiological mechanism of GvHD. However, as most of the treatment modalities are implemented in combinations, the optimal combination has not yet been identified. Moreover, some patients respond well to a decrease in the intensity of immunosuppressive treatment[38,55], or to replacement with another immunosuppressive agent[38,39,60,61], but good outcomes have also been reported using incremental doses of immunosuppressive drugs[52,57,58,62]. On the other hand, the literature has also reported the development of acute rejection in patients whose immunosuppressive drug dosage was decreased or the relevant medication was ceased; hence, switching to another medication may seem more reasonable than changing the dosage of the main immunosuppressive agent[31]. Each patient should be evaluated individually.

Among the most frequently administered treatment modalities for GvHD after LT mentioned in the literature are corticosteroid treatment[9,25,46,56], decrease/cessation/increase in or replacement of the immunosuppressive medication[22,26,52,55,62], and the use of antibodies directly targeting T lymphocytes, monoclonal antibodies targeting various receptors on the surfaces of lymphocytes, intravenous immunoglobulin[31,42,58,63,64] as an immune support, and antimicrobial treatments appropriate to suppress the infection[10,18].

Most of the experience regarding corticosteroid use in treating GvHD is based on the practices of hematopoietic stem cell transplantation[4]. The lympholytic and immunosuppressive effects of steroids, in addition to their potent anti-inflammatory characteristics, have provided justification for their widespread administration[4]. In our literature search, steroid treatment was instituted in 61 of 87 patients in whom GvHD developed after LT, whereas other treatment modalities were preferred in 21, and the remaining 5 patients were monitored for symptoms[19,38,65]. Death occurred in 43 patients on steroid treatment. Immunosuppressive treatment was re-administered upon development of acute rejection in two patients in whom the main immunosuppressive treatment was replaced by steroid treatment. Etanercept (Enbrel) therapy was commenced in one patient due to a failed response to steroid treatment, and a reduction in cyclosporine, and this approach yielded a successful outcome[45]. Most of the patients who experienced complications or a suboptimal response to treatment were administered various monoclonal antibodies or antagonist agents to T-lymphocytes[65-69]. The most commonly used drugs were the following: daclizumab (Zenapax)[21,29,32,68] and basiliximab (Simulect)[4,5,11,28,41], which bind to the CD25 subunit of interleukin (IL)-2 receptors on the surface of T-lymphocytes; muromonab (OKT3)[7,23,47,53], which binds to CD3 receptors on the surface of T-lymphocytes; alemtuzumab (Campath-1H)[50], which binds to CD52 receptors on the surface of mature lymphocytes; infliximab (Remicade)[30,32], which was developed against tumor necrosis factor-alpha; denileukin diftitox (Ontak)[39], which was developed by conjugation with diphtheria toxin for use against the IL-2 receptors on the surface of T-lymphocytes; and rituximab (Mabthera)[5], which binds to CD20 receptors on the surface of B lymphocytes. In addition to these agents, anti-thymocyte globulin (ATG)[26,28,66,67,69], effective directly on T-lymphocytes, and anti-lymphocyte globulin (ALG)[6,19,22] were also frequently utilized during treatment. In our literature analysis, we found that ATG, basiliximab, muromonab, ALG, daclizumab, infliximab, alemtuzumab, and rituximab and denileukin diftitox were administered in 25, 11, 7, 5, 4, 2, 1, 1, and 1 of the patients, respectively. Of these 57 patients, 13 were placed on monoclonal antibodies and/or T-lymphocyte antagonists as a first treatment modality, whereas steroids, immunosuppressive agents, and various combinations thereof were administered in 44 patients. Mortality rates did not differ among treatment conditions but were quite high in all treatment modalities, indicating that the most appropriate treatment modality has yet to be developed.

Prognosis of GvHD after liver transplantation

The prognosis for GvHD that develops after LT is rather poor, and mortality rates mentioned in the literature range from 75% to 91.6%[9,27,28,32,58]. The mortality rate observed in our literature analysis (67.8%) was lower than that reported in studies cited above. Nearly all patients died of multiple organ dysfunction syndrome, sepsis, or gastrointestinal bleeding despite significant antimicrobial and hematologic support. The only study evaluating mortality in GvHD after LT was a literature search conducted by Taylor *et al*[40] that included 51 cases. According to that study, rash and fever were identified as risk factors for mortality. We obtained different results (Table 1), which suggest that bone marrow (pancytopenia) and intestinal (diarrhea) involvement had a severe effect on mortality. However, the retrospective nature of this study, exclusion of some studies due to inadequate data, failure to obtain sufficient data regarding an HLA match, and the absence of a standardized treatment protocol were limiting factors. Such high rates of mortality despite any type of aggressive treatment revive the issue of protective precautions prior to LT.

Prevention of GvHD after liver transplantation

Preventing GvHD among patients undergoing LT is an important issue. Depletion of T-lymphocytes from the liver before transplantation would eliminate the risk of GvHD. This could be achieved, at least in principal, by

treating the cadaveric donor with ALG or by modifying the donor liver *ex vivo* by irradiation or perfusion with lytic monoclonal antibodies directed against a lymphocyte cell-surface protein[7,8]. However, whether these approaches can be justified is debatable, given the low incidence of GvHD after LT[40,47]. The donor's immunoactive cells can be removed by sufficient perfusion of the graft by carefully removing perihepatic lymph nodes or through graft radiation[31]. Based on our LT experience, perfusion of grafts from living or cadaveric donors with University of Wisconsin (Viaspan) or histidine-tryptophan-ketoglutarate (Custodiol) solution, followed by lactated Ringer's solution at 4 ℃, has proved a fairy efficacious method to remove donor-related lymphocytes from graft material. Some authors believe that transfusion-associated GvHD can be prevented by irradiating blood products and avoiding the use of related donors. Therefore, limiting the application of blood products and using washed red blood cells, white blood cell-free plasma, or platelets could contribute to the prevention of GvHD[31]. We prefer to irradiate erythrocyte suspensions routinely before transfusion in patients who have undergone LT.

In conclusion, although GvHD is a rare complication of LT and the mortality rate remains very high, clinical features represent an important tool for early diagnosis. The prognosis remains poor and further research is needed to clarify the pathogenesis of GvHD and to provide new therapeutic agents for treating this condition effectively.

COMMENTS

Background

Graft-versus-host disease (GvHD) occurs when the donor's immunocompetent cells react against the recipient's cellular antigens. GvHD is a well-known complication in patients who undergo bone-marrow transplantation. However, few reports have described GvHD after liver or other solid organ transplantation. The prognosis for GvHD after liver transplantation (LT) is rather poor, and mortality rates mentioned in the literature range from 75% to 91.6%. Therefore, it is important to determine the factors that affect the prognosis of the disease.

Research frontiers

The authors performed an extensive literature review regarding the development of GvHD after LT that included articles in the PubMed, Medline, EBSCO, EMBASE, and Google Scholar databases published between November 2011 and March 1988.

Innovations and breakthroughs

This study is the most extensive literature review examining factors affecting mortality in patients who develop GvHD after LT.

Applications

Univariate analyses showed that pancytopenia, diarrhea, an age difference between the recipient and donor, and time from first symptom occurrence to diagnosis or treatment were significant risk factors for mortality, and multivariate analysis demonstrated that an age difference between the recipient and donor was an independent risk factor for mortality.

Terminology

GvHD can be divided into acute and chronic forms depending on the timing and character of alloimmune activity. The acute form comprises all manifestations that occur during the first 100 d after transplantation, and the chronic form includes all manifestations that occur after 100 d. GvHD can also be divided into humoral and cellular forms. The humoral form is characterized by hemolysis and fever and occurs in patients transplanted with ABO-incompatible or non-identical grafts. The cellular form occurs when immunocompetent donor lymphocytes originating from the transplanted liver undergo activation and clonal expansion, allowing them to mount a destructive cellular immune response against recipient tissues.

Peer review

This is a good descriptive study in which authors determine the factors affecting mortality in patients who developed GvHD after LT. The results are interesting and showed that an age difference between the recipient and donor is an independent risk factor for mortality in patients who develop GvHD after LT.

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BRIEF ARTICLE

Short-term effectiveness of radiochemoembolization for selected hepatic metastases with a combination protocol

Shahram Akhlaghpoor, Alireza Aziz-Ahari, Mahasti Amoui, Shahnaz Tolooee, Hossein Poorbeigi, Shahab Sheybani

Shahram Akhlaghpoor, Department of Computed Tomography, Noor Medical Imaging Center, Sina Hospital, Tehran University of Medical Sciences, Tehran 15667-86553, Iran
Alireza Aziz-Ahari, Department of Radiology, Sina Hospital, Tehran University of Medical Sciences, Tehran 11367-46911, Iran
Mahasti Amoui, Department of Nuclear Medicine, Shohada-e-Tajrish Medical Center, Shahid Beheshti University of Medical Sciences, Tehran 19899-34148, Iran
Shahnaz Tolooee, Hossein Poorbeigi, Shahab Sheybani, Nuclear Science and Technology Institute, Tehran 14155-1239, Iran
Author contributions: Akhlaghpoor S designed the study, performed the procedures, and supervised the team; Aziz-Ahari A searched the literature, analyzed the data and drafted the manuscript; Amoui M planned the nuclear medicine aspects of the study; and Sheybani S, Tolooee S and Poorbeigi H designed and produced the radioisotope particles.
Correspondence to: Dr. Shahram Akhlaghpoor, MD, Department of Computed Tomography, Noor Medical Imaging Center, Sina Hospital, Tehran Medical University, Tehran 15667-86553, Iran. akhlaghpoor@nmri-ir.com
Telephone: +98-21-88086783 Fax: +98-21-88086782
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Abstract

AIM: To introduce the combination method of radiochemoembolization for the treatment of selected hepatic metastases.

METHODS: Twenty patients with biopsy proven hepatic metastases were selected from those who underwent transarterial radiochemoembolization, a novel combination protocol, between January 2009 and July 2010. Patients had different sources of liver metastasis. The treatment included transarterial administration of three chemotherapeutic drugs (mitomycin, doxorubicin and cisplatin), followed by embolization with large (50-150 μm) radioisotope particles of chromic 32P. Multiphasic computer tomography or computer tomography studies, with and without contrast medium injections, were performed for all patients for a short-term period before and after the treatment sessions. The short-term effectiveness of this procedure was evaluated by modified response evaluation criteria in solid tumors (mRECIST), which also takes necrosis into account. The subjective percentage of necrosis was also assessed. The response evaluation methods were based on the changes in size, number, and the enhancement patterns of the lesions between the pre- and post-treatment imaging studies.

RESULTS: Patients had liver metastasis from colorectal carcinomas, breast cancer, lung cancer and carcinoid tumors. The response rate based on the mRECIST criteria was 5% for complete response, 60% for partial response, 10% for stable disease, and 25% for progressive disease. Regarding the subjective necrosis percentage, 5% of patients had complete response, 50% had partial response, 25% had stable disease, and 20% had progressive disease. Based on traditional RECIST criteria, 3 patients (15%) had partial response, 13 patients (65%) had stable disease, and 4 patients (20%) had disease progression. In most patients, colorectal carcinoma was the source of metastasis (13 patients). Based on the mRECIST criteria, 8 out of these 13 patients had partial responses, while one remained stable, and 5 showed progressive disease. We also had 5 cases of breast cancer metastasis which mostly remained stable (4 cases), with only one partial response after the procedure. Six patients had bilobar involvement; three of them received two courses of radiochemoembolization. The follow up imaging study of these patients was performed after the second session. In the studied patients there was no evidence of extrahepatic occurrence, including pulmonary radioactive deposition, which was proven by Bremsstrahlung scintigraphy performed after the treatment sessions. For the short-term follow-ups for the 2 mo after the therapy, no treatment related death was reported. The mostly common side effect was post-embolization

syndrome, presented as vomiting, abdominal pain, and fever. Nineteen (95%) patients experienced this syndrome in different severities. Two patient had ascites (with pleural effusion in one patient) not related to hepatic failure. Moreover, no cases of acute liver failure, hepatic infarction, hepatic abscess, biliary necrosis, tumor rupture, surgical cholecystitis, or non-targeted gut embolization were reported. Systemic toxicities such as alopecia, marrow suppression, renal toxicity, or cardiac failure did not occur in our study group.

CONCLUSION: Radiochemoembolization is safe and effective for selected hepatic metastases in a short-term follow-up. Further studies are required to show the long-term effects and possible complications of this approach.

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Key words: Hepatic metastasis; Radiochemoembolization; Phosphorus radioisotopes; Treatment; Outcome

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INTRODUCTION

Although complete surgical resection of the hepatic portion affected by metastasis is usually the best treatment option, most patients with hepatic metastasis are not amenable to resection or have some contraindications to the surgery[1]. As alternatives to standard systemic chemotherapy, some recent palliative therapies have been developed for unresectable hepatic metastases, which include transarterial administration of chemotherapeutic drugs or radiopharmaceuticals, selective tumor vessel embolization and percutaneous tumor ablation with ethanol injection, cryotherapy, radiofrequency, or the use of microwaves[1,2].

Transcatheter arterial chemoembolization (TACE) is a dual minimally invasive therapeutic approach combining transarterial administration of chemotherapeutic drugs and hepatic artery embolization[3]. Although there are many advantages of this combination, it does produce marked tumoral ischemia at the time of drug administration which potentiates the effect of cytotoxic agents and augments tumoral cell apoptosis[4,5].

Radioembolization, on the other hand, is a technique that preferentially targets hepatic lesions by infusing the hepatic arteries that supply the tumor with radioactive microspheres[6]. Traversing the hepatic vascular plexus, these microspheres embed within the tumor arterioles, where they deliver high-energy low-penetrating radiation doses to the tumoral cells, while the normal hepatic tissue is relatively preserved[6,7]. As can be determined from the method's name, radioembolization has also microembolic effects and leads to subsequent vessel occlusion[8].

Regarding the effectiveness of radioembolization and chemoembolization for hepatic malignant neoplasms[2,9], we assumed that the local combination of the two methods (i.e., radiochemoembolization) would be more effective. It has been shown that radioembolization in combination with systemic chemotherapy is an effective first-line therapy for liver metastases[10]. The most commonly used agent for radioembolization of hepatic tumors is Yttrium-90 (^{90}Y) in the form of ^{90}Y microspheres[9], which has a half-life of 64 h[11]. In this study, however, a phosphorus-32 containing particle was adopted as the radiopharmaceutical. In the process of phosphorus-32 (^{32}P) decay, the molecule emits relatively high energy beta particles[12]. Although there are reports of safe clinical ^{32}P application for hepatic tumors[12-16], larger particles were used in the current study to reduce systemic toxicities even more by decreasing hepatic-to-systemic shunt. In addition, the higher half-life of ^{32}P (14.3 d[17]) would provide a longer irradiation time in order to achieve chemo-radiation effects.

The primary purpose of this study is to introduce the radiochemoembolization method for the treatment of hepatic metastases. Short-term effectiveness of this treatment based on imaging criteria was also assessed. As World Health Organization (WHO) criteria and response evaluation criteria in solid tumors (RECIST) guidelines are based solely on the degree of tumor shrinkage for assessing tumor response, we used other criteria like modified RECIST (mRECIST), which includes the degree of necrosis to show the effectiveness of the therapy[18]. Finally, *via* a brief review of the literature concerning ^{32}P application and TACE, possible limitations, concerns, and complications that may be encountered with radiochemoembolization were addressed.

MATERIALS AND METHODS

This was a single institution clinical study approved by the ethics committee of our imaging center. A written consent form was obtained from all patients and they were all informed about the novelty of the method. This paper reports the results of 20 patients who underwent radiochemoembolization between January 2009 and July 2010. The inclusion criteria included: biopsy proven hepatic metastatic lesion/lesions from any source; contraindication to ablative therapies and resection; an eastern cooperative oncology group performance status score of 0 to 2[19]; and the patient needed to be at least 18 years of age. Although more than 20 patients met these criteria and received radiochemoembolization, another inclusion criterion was added to only report the results of patients

who had available contrast-enhanced computer tomography (CT) or magnetic resonance imaging (MRI) 1 to 2 mo previous and after the treatment session. Only 20 patients such patients were qualified. Exclusion criteria were: bleeding diathesis that could not be controlled; significant extra-hepatic involvement, generally more than 50% of the whole tumoral bulk outside the liver; imminent threat to the patient's life caused by the disease; greater than 75% involvement of the hepatic parenchyma; severe hepatic dysfunction; and an active uncontrolled infection.

Patients fasted overnight and received a prophylactic antibiotic (ceftriaxone, 1 g) and antiemetics (granisetron, 3 mg; dexamethasone, 8 mg). During the procedure, fentanyl or pethidine were infused to alleviate the pain caused by embolization. All procedures were performed in the angiography room under aseptic conditions. Intravenous hydration was started 1 h before the procedure.

In this study, ^{32}P-containing particles were used (Nuclear Science and Technology Institute, Iran) with $Cr^{32}PO4$ as the active component. These particles had a grain size of 50-150 μm, significantly larger than previously used colloidal ^{32}P particles also based on $Cr^{32}PO4$[13,14,20]. The physical half-life period of ^{32}P is 14.28 d, with an average penetration distance of 3-4 mm in soft tissues (maximum 8 mm)[13]. Ranging from 0.185 to 0.444 GBq, the dose of injected solution was calculated based on liver volume (not tumor burden) which was estimated with CT or MRI. The prepared ^{32}P solution was dissolved in 1-3 mL of radiographic contrast. The chemotherapeutic mixture consisted of 50 to 100 mg of cisplatin, 50 mg of doxorubicin, and 8-10 mg of mitomycin-C dissolved in 10 mL of radiographic contrast and 10 mL of normal Saline.

Using the Seldinger technique, a catheter was introduced through the femoral artery and selective catheterization of the hepatic artery was performed. A 3-F hydrophilic microcatheter (Cook, United States) used with a 0.014 or 0.021 guide wire was suffice to catheterize the desired artery. This standard catheter allows rapid injection of viscous radiochemoembolic emulsions and is unlikely to clog with particles. A digital subtraction angiography was performed to confirm that there was no hepatic arteriovenous fistula or duodenogastric reflux. For patients who had bilobar involvement, the treatment mixture was infused in both lobes simultaneously or separately in two sessions (3-4 wk apart) depending on the patient's liver function test and number of metastases. Only in one case (case 4) was coil embolization of gastroduodenal artery was performed before radiochemoembolization.

After placing the catheter in a suitable location, the chemotherapeutic mixture was infused and continuously monitored *via* fluoroscopy to avoid reflux into the untargeted arterial bed. Following this step, again under fluoroscopic surveillance, chromic ^{32}P solution was infused for vessel occlusion. If reflux happened, the infusion would be paused until the arterial flow resumed and then restarted at a lower speed.

After the procedure, intravenous hydration, antibiotics, and antiemetic therapies were continued for 24 h and analgesics were supplied for control of pain as needed. All the patients were discharged on the day after the procedure. Oral antibiotics were continued for 5 d, as well as oral antiemetics and analgesics if needed. Twenty-four to 72 h after radiochemoembolization of hepatic tumors, bremsstrahlung scintigraphy was performed in all patients to document ^{32}P particles that accumulated in tumoral locations of the liver, and also to ensure that there were no extrahepatic radioactive deposits.

For evaluating the short-term effectiveness of radiochemoembolization by means of imaging studies, two scans, whether CT or magnetic resonance (MR), were performed 1 to 2 mo before and after the treatment session. CT examinations were performed using a multidetector scanner (Sensation 64, Siemens, Germany), with 5-mm sections (120 kV, 250 mAs). Triphasic liver imaging (including unenhanced, arterial and portal venous phase images) was acquired. Contrast-enhanced scans were performed after approximately 30 s in the arterial phase and after 70 s in the venous phase from the injection of the contrast agent iohexol (Omnipaque 350, Amersham Health; 125 mL at a rate of 3-5 mL/s). MR studies were performed using a 1.5 Tesla machine (Magnetom Symphony, Siemens, Germany). The protocol consisted of axial and coronal thin-section T2-weighted HASTE, axial unenhanced spoiled-T1-weighted gradient echo with fat suppression, and dynamic axial fat-suppressed contrast-enhanced spoiled-T1-weighted gradient-echo sequences for the arterial and venous phases (45, 60 and 90 s) and also the delayed phase (2-5 min) after contrast infusion. The contrast agent was gadopentetate dimeglumine (Magnevist, Berlex Pharmaceuticals, 20 mL), followed by 20 mL of saline flush.

Evaluation of tumor response to therapy was based on mRECIST criteria and subjective percentage of necrosis. Firstly, up to two hepatic target lesions were selected in pre-treatment imaging studies. Said lesions must (1) be capable of being accurately measured in at least one dimension as 1 cm or more; (2) be suitable for repeat measurement; and (3) have intratumoral enhancement after contrast injection. If more than two lesions could met these criteria, the one with larger enhancing portions would be selected. All other hepatic lesions were only recorded and not measured at the baseline; their presence or absence would be noted in the follow-up exams. A viable tumor was defined as a portion of the target lesion which had an uptake of the contrast agent in any phase of the contrast enhanced studies. For the mRECIST criteria, the change in the longest diameter of viable tumors was considered for the evaluation of the response to treatment. On the other hand, necrosis was a portion of the target lesions which remained without contrast enhancement. Based on these necrotic portions, a subjective percentage of necrosis was attributed to each target lesion. Every individual patient had a sum of longest viable tumor diameters and necrosis percentages of target lesions. According to the changes in these amounts, responses were categorized into complete response, partial response, stable disease and progressive disease (Table 1).

Table 1 Assessment of target lesion response

mRECIST		RECIST		Subjective percentage of necrosis	
CR	Disappearance of any intratumoral enhancement in all target lesions	CR	Disappearance of all target lesions	CR	Disappearance of any intratumoral enhancement in all target lesions
PR	At least a 30% decrease in the sum of diameters of viable target lesions	PR	At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum of the diameters of target lesions	PR	At least a 30% increase in the sum of target lesion necrosis percentages
SD	Any cases that do not qualify for either partial response or progressive disease	SD	Any cases that do not qualify for either partial response or progressive disease	SD	Any cases that do not qualify for either partial response or progressive disease
PD	An increase of at least 20% in the sum of the diameters of viable target lesions or new lesion appearance	PD	An increase of at least 20% in the sum of the diameters of target lesions or new lesion appearance	PD	A decrease of at least 20% in the sum of target lesion necrosis percentages or new lesion appearance

mRECIST: Modified response evaluation criteria in solid tumors; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Table 2 Description of lesions for each patient and local tumor control outcomes with regard to different criteria

No.	Sex	Age (yr)	No. of lesions	Location	Primary source	New lesion	mRECIST	RECIST	Necrosis	Sum of viable diameters (mm)		Sum of necrosis percentages (%)		Sum of diameters (mm)	
										Baseline	Post-procedural	Baseline	Post-procedural	Baseline	Post-procedural
1	F	41	Multi	U	BRE	No	PR	SD	PR	57	18	0	100	57	44
2	F	29	Multi	U	BRE	No	SD	SD	SD	34	34	5	5	34	34
3	F	35	1	U	BRE	No	PR	SD	PR	50	33	30	50	60	45
4	F	39	Multi	B	BRE	No	PR	SD	PR	57	39	20	100	65	54
5	F	38	Multi	B	BRE	Yes	PD	PR	PD	72	-[1]	30	-	72	-
6	F	27	2	U	CAR	No	CR	PR	CR	92	0	0	200	92	42
7	M	81	3	U	CRC	No	PR	SD	PR	73	23	40	95	107	95
8	M	40	1	U	CRC	No	PR	PR	SD	55	33	5	5	55	33
9	F	41	1	U	CRC	No	PR	SD	PR	65	45	40	80	80	68
10	F	47	1	U	CRC	No	PR	SD	PR	144	103	50	80	226	217
11	F	59	1	U	CRC	Yes	PD	PD	PD	70	-	0	-	70	-
12	M	60	Multi	U	CRC	Yes	PD	PD	PD	50	-	0	-	50	-
13	M	57	1	U	CRC	Yes	PD	PD	PD	25	-	70	-	35	-
14	M	55	1	U	CRC	No	PD	PD	SD	56	102	5	5	56	102
15	M	77	Multi	U	CRC	No	SD	SD	SD	107	107	15	15	116	116
16	F	62	Multi	B	CRC	No	PR	SD	SD	40	7	155	195	68	55
17	F	57	Multi	B	CRC	No	PR	SD	PR	50	35	90	180	65	55
18	M	74	Multi	B	CRC	No	PR	SD	PR	213	116	10	130	213	159
19	F	48	Multi	B	CRC	No	PR	SD	PR	245	160	5	120	265	260
20	F	51	Multi	U	LUN	No	PR	SD	PR	142	61	20	175	150	127

[1]For patients with new lesions in the follow-up scan, the measurements were not performed and the patient was marked as progressive disease (PD); CR: Complete response; PR: Partial response; SD: Stable disease; U: Unilobar; B: Bilobar; F: Female; M: Male; BRE: Breast cancer; CRC: Colorectal carcinoma; CAR: Carcinoid tumor; LUN: Lung cancer; mRECIST: Modified response evaluation criteria in solid tumors.

RESULTS

Patients had different sources of liver metastasis, but most were from colorectal cancer. None of our patients had a history of surgery for hepatic metastases. The demographic, clinical, radiological and response data of the studied patients are shown in Table 2.

The mean and median of the baseline total viable diameters (i.e., the sum of the maximum diameters of the viable portions of target lesions in each patient) were 84.85 mm and 61 mm (range: 25-245 mm), respectively. The response rate, based on mRECIST criteria, was 5% for complete response (Figure 1A and B), 60% for partial response (Figure 1C and D), 10% for stable disease, and 25% for progressive disease (Figure 1E and F).

The baseline sum of the estimated percentage of necrosis in target lesions was calculated for each patient, and had a mean and median of 29.5% and 17.5%, respectively (range: 0% to 155%). Regarding the necrosis percentage, 5% of patients had complete response, 50% had partial response, 25% had stable disease and 20% had progression. Based on traditional RECIST criteria 3 patients (15%) had partial response, 13 patients (65%) had stable disease, and 4 patients (20%) had disease progression.

In most patients, colorectal carcinoma was the source of metastasis (13 patients). Based on mRECIST, 8 out of these 13 patients had partial responses while one remained stable and 5 showed progressive disease. We also had 5 cases of metastasis from breast cancer, which mostly remained stable (4 cases) with only case of one partial response after the procedure.

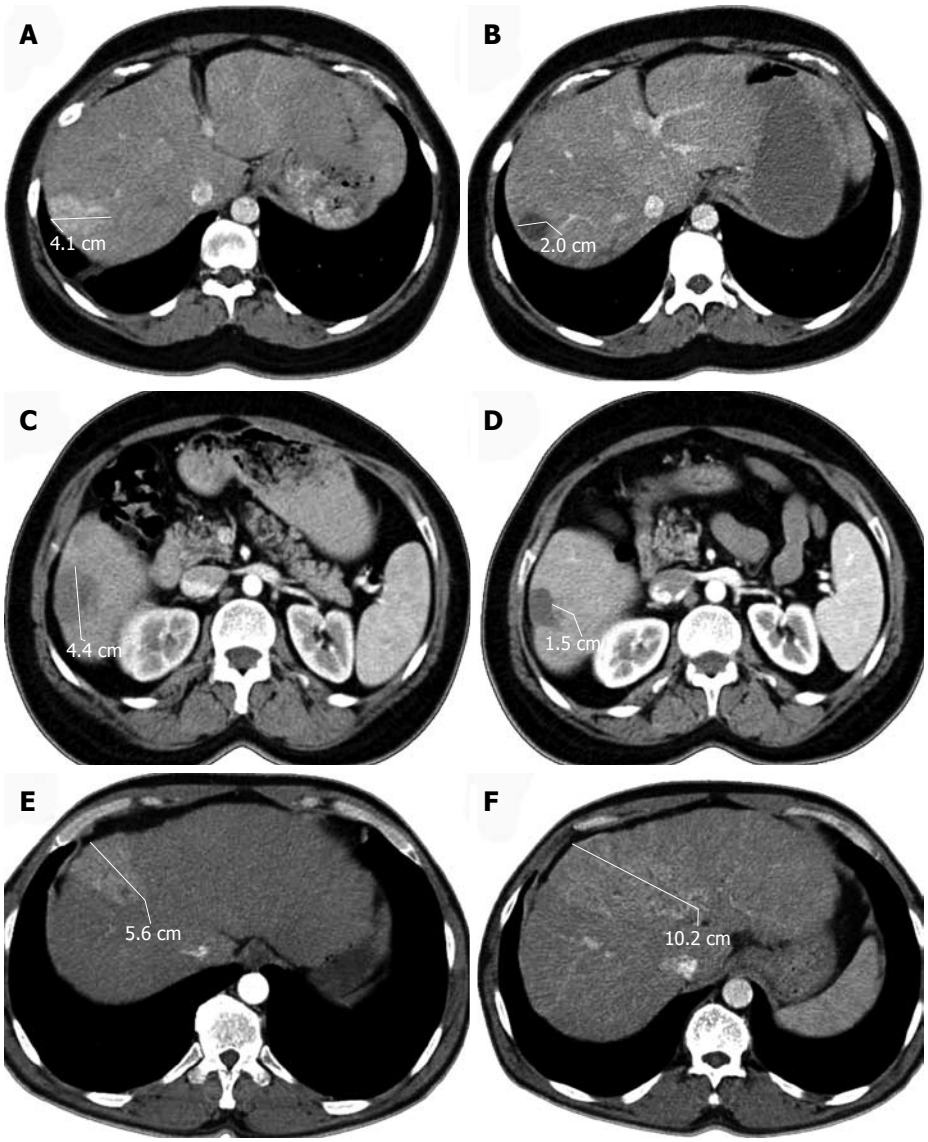


Figure 1 Radiochemoembolization images. A, B: Pre- (A) and post- (B) radiochemoembolization images in a 27 year-old female with a metastatic carcinoid tumor. There is no evidence of enhancing of the viable tumor after treatment. Based on modified response evaluation criteria in solid tumors (mRECIST) criteria, the response is complete, but regarding RECIST criteria we have a partial response; C, D: Pre- (C) and post- (D) radiochemoembolization images in a 41 year-old female with a metastatic carcinoid tumor. Based on modified response evaluation criteria in solid tumors criteria the response is partial; E, F: Pre- (E) and post- (F) radiochemoembolization images in a 55 year-old male with a metastatic colorectal carcinoma. Based on modified response evaluation criteria in solid tumors criteria the response is progressive.

Six patients had bilobar involvement, with three of them receiving two courses of radiochemoembolization. The follow-up imaging study of these patients was performed after the second session. In the studied patients there was no evidence of extrahepatic occurrence (such as pulmonary radioactive deposition), which was proven by Bremsstrahlung scintigraphy performed after the treatment.

For short-term follow-ups for the 2 mo after the therapy, no treatment-related death was reported. The most common side effect was post-embolization syndrome, presented as vomiting, abdominal pain and fever. Nineteen (95%) patients experienced this syndrome in different severities. Two patient had ascites (with pleural effusion for one patient) not related to hepatic failure. Moreover, no cases of acute liver failure, hepatic infarction, hepatic abscess, biliary necrosis, tumor rupture, surgical cholecystitis, or non-targeted gut embolization were reported[2]. Systemic toxicities, such as alopecia, marrow suppression, renal toxicity, or cardiac failure did not occur in our study group.

DISCUSSION

Liver metastases are one of the most difficult therapeutic challenges in oncological management, and are not usually amenable to resection. Many studies have been performed to find more effective palliative options for non-operable metastatic tumors. Although systemic chemotherapy still has a role[10,21], there are attempts at focusing treatment on hepatic tumors[22]. The present study introduced a novel combination of two effective treatment

options, TACE and radioembolization, for metastatic hepatic lesions.

In the current study, large-molecule chromic phosphate containing ^{32}P particles were used. Colloidal ^{32}P, another chromic phosphate-containing agent, has been previously used for radiosynovectomy *via* intrasynovial injection[23], treatment of stage I and II ovarian carcinoma *via* intraperitoneal instillation[24,25], and for regional radiotherapy of some tumors *via* direct intratumoral injection[13,20]. The usual forms of colloidal ^{32}P particles are small, approximately 1 μm[14,26], and might leak into systemic circulation, causing irradiation to undesired parts of body and toxicity. For intrasynovial and intraperitoneal application, as the risk of leakage is low, the colloidal ^{32}P solution is safely used[23]. For direct intratumoral injection on the other hand, the retention of colloidal ^{32}P radioactivity at the site of a solid tumor requires the co-administration of macroaggregated albumin[27]. However, the risk of leakage from the injection site is still present due to intratumoral interstitial pressure[14].

There was only one report of intravascular injection of ^{32}P colloid in our literature review. Kim *et al*[28] administered colloidal ^{32}P *via* the portal vein to prevent growth of occult metastases in the liver. They concluded that the mentioned approach would be expected to prevent liver metastases of completely resected colorectal cancers. Other studies on radioactive phosphorus use phosphorus-32 glass microspheres (^{32}P-GMS) with grain sizes of 46-76 μm to reduce systemic toxicity[13]. Although transarterial administration of this compound has been used safely for hepatic primary or metastatic tumors[13,15,16], the main use for a radioactive pharmaceutical for this purpose is a ^{90}Y microsphere with a particle size of 20-35 μm[9]. There has also been an early report of ^{90}Y systemic leakage[29].

In terms of systemic toxicity, the used compound did not have a higher risk than ^{32}P-GMS or ^{90}Y microspheres. We can, however, raise an advantage for ^{32}P over ^{90}Y regarding our purpose. The half life of this radioactive element (14.28 d) provides a significantly longer period of irradiation than ^{90}Y with its half-life of 64 h[11,13]. Considering the 2 half-lives, there was almost 28 d of radiation for the optimal chemoradiation effect in the presence of chemotherapy drugs.

Another consideration is our chemotherapeutic mixture. Although there is no consensus on the best chemotherapeutic agent for TACE, doxorubicin is the most commonly used drug for the purpose[30]. The most commonly combined drug regimen for TACE, including cisplatin, doxorubicin, and mitomycin C[2,30] was used in this study. In combination with radiotherapy, however, we needed to find some supports and check if there were previous contraindications in the literature. Cisplatin is similar to other platinum-based agents that act as a radiosensitizer[31]. There was one clinical trial combining hepatic radioembolization with ^{90}Y and a systemic chemotherapy regimen containing the platinum-based agent, oxaliplatin[32]. Concerning doxorubicin, which is also a potent radiosensitizer[33], we found no previous usage for hepatic malignancies in combination with radiotherapy, although its co-administration has been used in other body parts[34-36]. Another study on nude mice for medullary thyroid cancer showed the combination of radioimmunotherapy and doxorubicin chemotherapy had synergistic therapeutic efficacy, which may be due to the radiosensitizing effect of doxorubicin[37]. Like doxorubicin, mitomycin C has also had concurrent administration with radiotherapy in several studies[38-40]. Therefore, there is no proven contraindication to applying radiotherapy along with this chemotherapeutic regimen. Moreover, it is expected that in the case of cisplatin and doxorubicin, which are radiosensitizers, the effect of therapy would be more effective than just the addition of TACE and radioembolization effects.

The mRECIST criteria were originally designed for hepatocellular carcinoma (HCC) and are based on the changes in the viable portion of hepatic lesions. Older methods of image-based response evaluation of solid tumors only assess the change in anatomic size of target lesions[18]. Measurements were either by the bilinear product approach (WHO criteria) or by single linear summation (RECIST criteria)[41]. As acknowledged before, relying solely on the changes in tumor size can be misleading[42]. Modified RECIST and a subjective percentage of necrosis criteria take tumor necrosis induced by treatment into account[18].

Studies which used ^{90}Y radioembolization for metastatic hepatic lesions from mixed sources generally relied on WHO and RECIST criteria to assess the treatment response (Table 3). Expanding the response rate to cases with complete or partial response, there were reports of 13% to 42.8% responsiveness with regard to WHO and RECIST criteria. Only Peynircioğlu *et al*[43] reported that all of their patients had at least a partial response in target lesions. Considering necrosis in combination with anatomic size, Miller *et al*[7] showed an increase in response rate from 19% to 50%. The studies on metastatic hepatic lesions using a chemotherapy regimen of doxorubicin, cisplatin, and mitomycin-c for TACE are summarized in Table 4. Only papers which reported an imaging-based response rate were included. WHO and RECIST criteria showed a response rate that differed from 8% to 60% in these studies. A paper by Artinyan *et al*[44] on mixed-source hepatic metastases showed a response rate of 14.8%.

Firusian *et al*[20] reported 5 cases of hepatic metastasis for which direct intratumoral colloidal ^{32}P injection led to three complete and two partial responses. No toxicity was encountered in these 5 patients and there were no alterations in hepatic function. In a study by Gao *et al*[13] on 60 patients with refractory solid tumors, including 25 cases of HCC and 5 cases of hepatic metastatic carcinoma, they administered ^{32}P-GMS *via* the hepatic artery for thirty-two cases. Among all 60 patients, 31 cases achieved complete response (51.7%), 25 cases partial response (41.7%) and 4 cases no effect. Most patients had post-procedural nausea and vomiting. There were also reports of discomfort or pain in the right upper abdominal quad-

Table 3 Studies on Yttrium-90 radioembolization for metastatic hepatic lesions from mixed sources

Study	Procedure	Agent	Absorbed dose or mean activity delivered[1]	Number of patients	Response criteria	Response measured at (months post treatment)	Response rate	Complications
Blanchard *et al*[49], 1989	Radioembolization	^{90}Y plastic microspheres	NA	15	WHO	NA	Partial response in 5 (33.3%), minimal response in 2 (13.3%)	Gastritis or gastric ulceration in 6 (in three this was proven to be due to unintended infusion of microspheres into the gastric circulation)
Andrews *et al*[50], 1994	Radioembolization	^{90}Y glass microspheres	150 Gy	24	WHO	2	Partial response in 5 (20.8%), minimal response in 4 (16.7%), stable disease in 7 (29.2%), progressive disease in 8 (33.3%)	Mild gastrointestinal symptoms in 4 (unrelated to treatment)
Miller *et al*[7], 2007	Radioembolization	^{90}Y glass microspheres	100-120 Gy	42	WHO	2.3[2]	Complete/partial response in 8 (19%), stable disease in 22 (52%), progressive disease in 23	Radiation cholecystitis in 10, liver edema in 18
					RECIST	3.9[2]	Complete/partial response in 10 (24%), stable disease in 21 (50%), progressive disease in 23	
					Necrosis	1[2]	Complete/partial response in 19 (45%)	
					Combined	1.1[2]	Complete/partial response in 21 (50%), stable disease in 11 (26%)	
Sato *et al*[8], 2008	Radioembolization	^{90}Y glass microspheres	112.8 Gy/ 1.83 GBq	137	WHO	1-3	Complete response (2.1%), partial response (40.7%)	Fatigue (56%), vague abdominal pain (26%), nausea (23%)
Lim *et al*[51], 2005	Radioembolization	^{90}Y resin microspheres	NA	46	RECIST	2	Partial response in 12 (27%), stable disease in 12 (27%), progressive disease in 19 (44%)	Between 2 and 8 wk of lethargy, anorexia, nausea and right upper quadrant pain in most patients, severe gastric/duodenal ulceration in 4 (8%), portal hypertension in 1, radiation hepatitis in 1
Yu *et al*[52], 2006	Radioembolization	^{90}Y resin microspheres	42 Gy	49	RECIST	NA	Response rate of 29%	Fatigue in 18 (37%), vague abdominal pain in 10 (20%), nausea/vomiting in 10 (20%), ascites and/or leg edema in 3 (6%)
Szyszko *et al*[53], 2007	Radioembolization	^{90}Y resin microspheres	1.9 GBq	21	RECIST	1-2	Partial response in 2 (13%), stable disease in 9 (60%), progressive disease in 4 (27%)	NA
Stuart *et al*[54], 2008	Radioembolization	^{90}Y resin microspheres	NA	30	RECIST	NA	Partial response or stable disease in 14 (47%)	Gastrointestinal ulceration in 1 (3%)
Kennedy *et al*[55], 2009	Radioembolization	^{90}Y resin microspheres	1.1 ± 0.6 GBq	502[3]	RECIST	3	Complete response in 23 (4.5%), partial response in 48 (9.5%), stable disease in 386 (76.8%), progressive disease in 45 (9%)	Fatigue and upper abdominal pain (29%), gastritis and overt gastric ulceration (2%), severe liver disease (4%)
Peynircioğlu *et al*[43], 2010	Radioembolization	90Y resin microspheres	1.24 GBq	10	RECIST	1-2	All patients had at least partial response of the target lesions	Post-procedural mild to moderate fatigue in all patients for 7 d, with mild to moderate fever and abdominal pain in some patients
Omed *et al*[56], 2010	Radioembolization	^{90}Y resin microspheres	NA	11	RECIST	NA	Partial response (20%), stable disease (50%), progressive disease (30%)	No major complications, 82% of patients experienced side-effects, mainly nausea, vomiting and abdominal pain
Cianni *et al*[57], 2010	Radioembolization	^{90}Y resin microspheres	1.64 Gbq	110	RECIST	2	Complete/partial response in 45, stable disease in 42, progressive disease in 23	Hepatic failure in 1, gastritis in 6, cholecystitis in 2

[1]The absorbed dose in Gy and/or the mean delivered activity in Gbq are provided with respect to their availability; [2]median; [3]the total number of patients in the study was 680, but the response evaluation criteria in solid tumor (RECIST) criteria were only available for 502 patients. ^{92}Y: Yttrium-90; WHO: World Health Organization; NA: Not available.

rant within 1 wk after treatment[13].

There are significant differences between lesion outcomes rated by the mRECIST and RECIST criteria in our series. In agreement with many other reports, in short-term follow-up the degree of necrosis is a major factor for response evaluation and a criteria lacking this factor may

Table 4 Studies on chemoembolization for metastatic hepatic lesions with cisplatin, doxorubicin and mitomycin

Study	Primary diagnosis	Procedure	Chemotherapeutic agents	Embolic material	Number of patients	Response criteria	Response measured at months treatment	Response rate	Complications
Diaco *et al*[58], 1995	Carcinoid tumor	Chemoembolization	Cisplatin, doxorubicin, mitomycin	NA	10	WHO	NA	Partial response (60%), stable disease (30%)	NA
Drougas *et al*[59], 1998	Carcinoid tumor	Chemoembolization	Doxorubicin (60 mg), cisplatin (100 mg), and mitomycin (30 mg)	Polyvinyl alcohol	13[1]	WHO	3	Partial response in 1 (8%), minimal response in 10 (77%), stable disease in 1 (8%), progressive disease in 1 (8%)	Nausea/vomiting in 100%, increased transaminases in 100%, pain in 100%, fever in 29%, myelosuppression in 29%, arterial thrombosis in 8%, dysrhythmia in 8%, mental status changes in 4%
Tellez *et al*[60], 1998	Colorectal carcinoma	Chemoembolization	Cisplatin, doxorubicin, mitomycin	Angiostat (a bovine collagen material)	27	Designed by authors[2]	NA	Radiological response in 17 of 27 patients (63%)	Fever in 83%, RUQ pain in 100%, nausea/vomiting in 83%, gastritis in 17%, lethargy in 60%
Buijs *et al*[45], 2007	Breast cancer	Chemoembolization	Doxorubicin (50 mg), cisplatin (100 mg), and mitomycin (10 mg) in a 1:1 mixture with iodized oil	300- to 500-μm embolic microspheres	14[3]	RECIST	1-2	No complete response, partial response in 7 lesions (26%)[6]	NA
Ruutiainen *et al*[61], 2007	Neuroendocrine tumor	Chemoembolization	Cisplatin, doxorubicin, mitomycin in a mixture with iodized oil	150- to 250-μm granular polyvinyl alcohol particles	44	RECIST	1	88% partial response/stable disease	High incidence of postembolization syndrome, severe pain in 3 sessions, severe nausea in 1 session, severe vomiting in 1 session, severe GGT/ALP elevation in 4 sessions, severe AST elevation in 1 session, severe ALT elevation in 1 session, severe infection in 1 session
Artinyan *et al*[44], 2008	Mixed	Chemoembolization	Doxorubicin (50 mg), mitomycin (10 mg), and cisplatin (150 mg)	Polyvinyl alcohol microspheres (300-700 μm)	61[4]	RECIST	At least 1	Partial response in 9 (14.8%), progressive disease in 3 (4.9%)	Bleeding in 2 patients (2%), renal failure in 6 patients (5%), hepatic failure in 7 patients (6%), infection in 3 patients (3%), mortality in 30 d in 7 patients (6%)
Buijs *et al*[48], 2008	Ocular melanoma	Chemoembolization	Doxorubicin (50 mg), cisplatin (100 mg), and mitomycin (10 mg) in a 1:1 mixture with iodized oil	300- to 500-μm embolic microspheres	6[5]	RECIST	1-2	No complete response, partial response in 8 lesions[6]	NA
Albert *et al*[62], 2011	Colorectal carcinoma	Chemoembolization	Cisplatin, doxorubicin, mitomycin in a mixture with ethiodized oil	Polyvinyl alcohol	95[6]	RECIST	NA	Partial response in 9 (14.8%), stable disease in 49 (80.3%), progressive disease in 3 (4.9%)	NA

[1]There were 15 patients at first, however one died before the follow up imaging and one patient's follow-up was out of state. Three patients only received hepatic artery embolization; [2]at least 75% decrease in the density of lesions consistent with necrosis or 25% decrease in the size of the lesions without the development of concomitant lesions; [3]twenty-seven lesions; [4]the total number of patients was 119, but for 61 patients the response evaluation criteria in solid tumors (RECIST) criteria were available; [5]twenty-one lesions, reporting by patient, all patients were considered non-responders to transcatheter arterial chemoembolization because the total tumor burden did not decrease by 30% in any given patient; [6]ninety-five of 141 treatment cycles were evaluable for response. The response rate is calculated per treatment cycle. NA: Not available; RUQ: Right upper quadrant; WHO: World Health Organization; GGT/ALP: Gamma-glutamyl transpeptidase/alkaline phosphatase; AST/ALT: Aspartate aminotransferase/alanine aminotransferase.

underestimate the effectiveness of the therapy[7,42,45-48]. There were differences in the results of mRECIST and necrosis percentage criteria for 3 patients. In these cases the change was in the anatomical size of lesions without a significant change in necrosis percentage. This finding shows that, in addition to its inherent flaw of being subjective, necrosis percentage cannot always reveal the real response. It seems that criteria that gather both anatomic sizes and the degree of necrosis (e.g., mRECIST) are more accurate. In 2 patients with newly appeared lesions (cases 12 and 13) after the treatment, these new lesions were in the lobe other than the one that underwent radiochemoembolization. These lesions could be new metastatic lesions or previously non-visible metastatic

foci in the non-treated lobe. For that reason, assessing the response by patient and not by lobe might have shown lower response rates in this study.

Our patients, like in most other studies on radioembolization and TACE, generally had constitutional and mild gastrointestinal symptoms after treatment sessions. There was no severe toxicity in the short-term follow-up of our series. Various degrees and severities of complications have been previously encountered after TACE and radioembolization procedures (Tables 3 and 4).

One limitation of the current study is the lack of control groups which only receive either TACE or radioembolization. Therefore, statistical comparison between the methods is impossible. However, many studies in this field perform new treatment strategies without a sample group and compare their result with the literature (Tables 3 and 4). Other weaknesses of this study were the short-term follow-up period and the mixed sources of hepatic metastases. The metastatic foci from different sources may have different responses to the administrated therapy. Longer than 2 mo period imaging results, long-term survival rates, time to progression (i.e., the post procedural elapsed time after which imaging studies show progressive disease), and also response rates in larger series of patients with single source of metastasis remain to be reported on the forthcoming steps after completion of regular long-term imaging evaluations and follow-ups.

In conclusion, this study introduces a new treatment approach for hepatic metastatic lesions on a rational basis. This was a combination of TACE and radioembolization which have been used individually for such lesions. It also shows that in short-term follow-ups this method is safe and effective, with a response rate of 65% with regards to the mRECIST criteria. Further studies are required to show the long-term effects and possible complications of this approach.

COMMENTS

Background

Liver metastases are a therapeutic challenge in oncological management. Surgery is frequently impossible due to disease extent and systemic chemotherapy usually fails. Transcatheter arterial chemoembolization and radioembolization are two separately used locoregional palliative therapies for metastatic hepatic lesions.

Research frontiers

Designing new treatments for patients with multiple or unresectable liver metastases is an interesting field of oncology. Transcatheter arterial administration of therapies is rapidly developing with the aid of diverse chemotherapeutic drugs and radiopharmaceuticals. Application of microsphere and particle technology is an evolving area of interventional oncology.

Innovations and breakthroughs

This study presents a novel image-guided combination of transcatheter arterial chemoembolization and radioembolization for advanced hepatic metastases, referred to as radiochemoembolization, which substantially intensifies local treatment effect. The authors used chromic phosphorus-32 molecules embedded in large particles with greater local effects and less systemic toxicities.

Applications

By showing the short-term effectiveness of the new combination method, the study opens the way for further research studies to assess the effects and complications of radiochemoembolization more thoroughly. After the release of results from the ongoing and upcoming studies by the authors and other scientists, an adjusted form of radiochemoembolization might play an important role in the treatment of hepatic metastases.

Terminology

Radiochemoembolization is a newly designed combination of radioembolization and transcatheter arterial chemoembolization, which are minimally invasive therapeutic approaches for administrating radioactive microspheres and chemotherapeutic drugs transarterially.

Peer review

This paper presents the short-term effectiveness of radiochemoembolization for selected hepatic metastases. This is the first report in which the novel combination of two commonly used effective options of treatment is described.

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BRIEF ARTICLE

Adenosine deaminase activity in tuberculous peritonitis among patients with underlying liver cirrhosis

Yi-Jun Liao, Chun-Ying Wu, Shou-Wu Lee, Chia-Ling Lee, Sheng-Shun Yang, Chi-Sen Chang, Teng-Yu Lee

Yi-Jun Liao, Chun-Ying Wu, Shou-Wu Lee, Sheng-Shun Yang, Chi-Sen Chang, Teng-Yu Lee, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung 407, Taiwan, China
Chun-Ying Wu, Teng-Yu Lee, Graduate Institute of Clinical Medical Science, China Medical University, Taichung 407, Taiwan, China
Shou-Wu Lee, Sheng-Shun Yang, Chi-Sen Chang, Teng-Yu Lee, Department of Medicine, Chung Shan Medical University, Taichung 407, Taiwan, China
Chia-Ling Lee, Medical Record Room, Taichung Veterans General Hospital, Taichung 407, Taiwan, China
Author contributions: Liao YJ, Wu CY and Lee TY designed this study; Lee CL provided data from medical records; Lee SW and Yang SS collected clinical data; Chang CS provided executive support for this work; and Liao YJ and Lee TY wrote the manuscript.
Correspondence to: Dr. Teng-Yu Lee, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Taichung Veterans General Hospital, 160, Section 3, Taichung Harbor Road, Taichung 407, Taiwan, China. tylee@vghtc.gov.tw
Telephone: +886-4-23592525 Fax: +886-4-23741331
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Abstract

AIM: To investigate the value of adenosine deaminase (ADA) for early detection of tuberculous peritonitis (TBP) among cirrhotic patients.

METHODS: We retrospectively analyzed 22 patients with TBP from July 1990 to June 2010. Twenty-five cirrhotic patients with uninfected ascites were prospectively enrolled as the cirrhosis control group from July 2010 to June 2011. An additional group of 217 patients whose ascites ADA levels were checked in various clinical conditions were reviewed from July 2008 to June 2010 as the validation group.

RESULTS: The mean ascites ADA value of cirrhotic patients with TBP (cirrhotic TBP group, n = 8) was not significantly different from that of non-cirrhotic patients (non-cirrhotic TBP group, n = 14; 58.1 ± 18.8 U/L *vs* 70.6 ± 29.8 U/L, P = 0.29), but the mean ascites ADA value of the cirrhotic TBP group was significantly higher than that of the cirrhosis control group (58.1 ± 18.8 U/L *vs* 7.0 ± 3.7 U/L, P < 0.001). ADA values were correlated with total protein values (r = 0.909, P < 0.001). Using 27 U/L as the cut-off value of ADA, the sensitivity and specificity were 100% and 93.3%, respectively, for detecting TBP in the validation group.

CONCLUSION: Even with lower ADA activity in ascites among cirrhotic patients, ADA values were significantly elevated during TBP, indicating that ADA can still be a valuable diagnostic tool.

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Key words: Extrapulmonary; Tuberculosis; Ascites; Cirrhosis; Peritonitis

Peer reviewers: Dr. Nimer Assy, Liver Unit, Ziv Medical Centre, 13100 Safed, Israel; A Ibrahim Amin, MD, Department of Surgery, Queen Margaret Hospital, Dunfermline, Fife KY12 0SU, United Kingdom

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INTRODUCTION

Tuberculous peritonitis (TBP) is one of the most frequent extra-pulmonary locations of tuberculosis, and the mortality rate may exceed 50% without prompt treatment[1,2]. The gold standard for diagnosing TBP is culture

of *Mycobacterium* in ascites fluid or peritoneal biopsy, but the cultures are time-consuming and have low positivity rates, with a mean sensitivity of 43%-83% depending on the quality of samples cultured and methods utilized[3,4]. In addition, caseous granulomas of peritoneal biopsies obtained by either laparoscopy or laparotomy are alternative methods for rapid primitive diagnosis, but the procedures are invasive and may increase rates of morbidity and mortality[4-6]. The high mortality rate in untreated patients warrants a quick and noninvasive test for diagnosing TBP[1,3,7], and adenosine deaminase (ADA) in ascites is an available test that has been proposed to be useful for rapid diagnosis[1,4,6,8,9]. By analyzing ADA level in ascites, the sensitivity and specificity for diagnosing TBP have been reported to be as high as 100% and 97%, respectively[2,8,10-12].

The risk of TBP is increased in patients with underlying liver cirrhosis[4,13], and the percentage of underlying cirrhosis among patients with TBP could be as high as 50% in the United States[14]. However, concerns regarding the sensitivity of ascites ADA in detecting TBP have been raised among patients with underlying liver cirrhosis[3,15], and the low sensitivity is thought to be caused by the concomitant immunocompromised status and dilutional phenomenon in advanced liver disease[3]. In addition, cirrhotic patients always have a low protein level in ascites, and the correlation between total protein and ADA activity has been discussed in previous studies[3,10,15]. However, the laboratory analysis of ascites might be confounded by heterogenous clinical conditions in the control groups, and previous studies show conflicting results for cirrhotic patients with TBP[4]. Therefore, in the present study, we employed a prospectively-enrolled cirrhosis control group in order to investigate the laboratory changes in ascites and the clinical utility of measuring ADA activity in cirrhotic patients with TBP.

MATERIALS AND METHODS

Retrospective tuberculous peritonitis cohort

All patients diagnosed with TBP at Taichung Veterans General Hospital, a tertiary referral center in central Taiwan, from July 1990 to June 2010 were retrospectively analyzed and formed the TBP group. The patients' medical records were reviewed and demographic, laboratory, microbiological, histological and laparoscopic features were collected. A definite diagnosis of TBP was based on one or more of the following: (1) positive TB cultures of ascites or peritoneal biopsy; (2) characteristic finding of caseous granulomas on histology of peritoneal biopsy; and (3) clinical judgment of TBP by a physician based on ascites data followed by a good response to anti-tuberculous treatment. A good response to therapy was defined as complete resolution or clinical improvement during the follow-up period. Exclusion criteria included (1) ascites ADA data unavailable; (2) ascites ADA analyzed after anti-tuberculous treatment; and (3) patients with concomitant end-stage renal disease under continuous ambulatory peritoneal dialysis, whose ascites might be diluted by dialysate. In addition, for further analysis of the role of liver cirrhosis, patients were evaluated to determine whether or not they had concurrent liver cirrhosis. Liver cirrhosis was defined as typical morphologic change such as blunted edge of liver, irregular liver surface and highly coarse liver parenchyma[16], with or without evidence of portal hypertension, such as previous history of ascites before TBP, splenomegaly or esophageal/gastric varices recognized by imaging techniques or endoscopy.

Prospective control cohort of cirrhosis

Patients with liver cirrhosis and uninfected ascites were prospectively enrolled as the cirrhosis control group from July 2010 to June 2011, and medical history, etiology of cirrhosis, and Child-Pugh's classification, as well as imaging features were recorded. Patients with primary or metastatic liver malignancy, peritoneal carcinomatosis, congestive heart failure, spontaneous bacterial peritonitis, nephritic syndrome, renal failure under dialysis, or evidence of peritonitis were excluded. Ascites was obtained and analyzed with regard to parameters including ADA, albumin, total protein, lactate dehydrogenase, glucose, cell counts, cytology, ordinary and anaerobic culture, acid-fast stain, bacteria and tuberculosis cultures.

Validation cohort

Patients whose ascites ADA levels were detected by a clinical need for differential diagnosis from July 2008 to June 2010 comprised the validation group and their medical records were retrospectively reviewed. The patients in the cirrhosis control group were not included in the validation cohort. Further analysis of subgroups according to etiology of ascites was performed.

Method of adenosine deaminase determination

ADA activity was determined using a method similar to that described by Slaats *et al*[17] with an autoanalyzer (Hitachi 7170, Japan) and ADA-N kit (Denka Seiken Co. Ltd, Japan). The kinetic method estimated ADA activity by coupling the liberated NH_3 to oxoglutarate with glutamate dehydrogenase, which leads to a decrease in the reduced form of nicotinamide adenine dinucleotide phosphate absorbance at 340 nm[3,17]. The ratio of decreased absorbance reflects the activity of ADA.

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by the Institutional Review Board of Taichung Veterans General Hospital (C10124).

Statistical analysis

The discrete variables are presented with number and percentage; continuous variables are presented with mean ± SD. The continuous variables were compared by Mann-Whitney *U* test. The discrete variables were compared by

Table 1 Comparisons between the tuberculous peritonitis without and with cirrhosis group

Demographic data	TBP without cirrhosis (n = 14)	TBP with cirrhosis (n = 8)	P value
Age (yr)	63.3 ± 22.8	66.5 ± 9.7	0.65
Sex			0.64
Male	10 (71.4)	5 (62.5)	
Female	4 (28.6)	3 (37.5	
Ascites data			
ADA (U/L)	70.6 ± 29.8	58.1 ± 18.8	0.29
SAAG (g/dL)	0.9 ± 0.3	1.3 ± 0.6	0.10
Total protein (mg/dL)	4781.8 ± 1645.5	3400.0 ± 1000.0	0.04
WBC (/mm^3)	1411.1 ± 1291.2	1489.9 ± 759.3	0.42
Lymphocytes (/mm^3)	1078.3 ± 935.6	1135.0 ± 696.1	0.71

Data are presented as mean ± SD or n (%). TBP: Tuberculous peritonitis; ADA: Adenosine deaminase; SAAG: Serum ascites albumin gradient; WBC: White blood cells.

Table 2 Comparisons between the tuberculous peritonitis with cirrhosis group and the cirrhosis control group

Demographic data	TBP with cirrhosis (n = 8)	Cirrhosis control (n = 25)	P value
Age (yr)	66.5 ± 9.7	59.8 ± 13.5	0.65
Sex			1.00
Male	5 (62.5)	15 (60.0)	
Female	3 (37.5)	10 (40.0)	
Child's classification			0.99
A	1 (12.5)	3 (12.0)	
B	4 (50.0)	12 (48.0)	
C	3 (37.5)	10 (40.0)	
Ascites data			
ADA (U/L)	58.1 ± 18.8	7.0 ± 3.7	< 0.001
SAAG (g/dL)	1.3 ± 0.6	2.1 ± 0.5	0.01
Total protein (mg/dL)	3400.0 ± 1000.0	1176.0 ± 636.6	< 0.001
WBC (/mm^3)	1489.9 ± 759.3	174.7 ± 159.6	< 0.001
Lymphocytes (/mm^3)	1135.0 ± 696.1	113.3 ± 127.1	< 0.001

Data are presented as mean ± SD or n (%). TBP: Tuberculous peritonitis; ADA: Adenosine deaminase; SAAG: Serum ascites albumin gradient; WBC: White blood cells.

χ^2 test and Fisher's exact test. Diagnostic utility of ADA for TBP was evaluated at various cutoff values by the sensitivity and specificity. These were assessed based on comparisons of relative operating characteristic curves. Spearman's linear regression was used to evaluate the correlation between total protein of ascites and serum. $P < 0.05$ was considered to be statistically significant.

RESULTS

A computerized database search identified 29 consecutive patients who were diagnosed with TBP from July 1988 to June 2010 and who met the inclusion criteria. Seven patients (6 patients without ADA data and 1 patient under continuous ambulatory peritoneal dialysis) were excluded according to the exclusion criteria of this study, and none had concurrent liver cirrhosis. A total of 22 patients with TBP were included in the final analysis and formed the TBP group, which was further divided into two subgroups: (1) cirrhotic TBP group: concomitant cirrhosis was diagnosed in 8 of 22 patients; and (2) non-cirrhotic TBP group: no evidence of cirrhosis among the other 14 of 22 patients. The demographic and ascites data of the two TBP subgroups were compared (Table 1), and there were no significant differences in ascites ADA, white blood cell (WBC) count and lymphocyte count between the two subgroups. However, the mean total protein concentration of ascites in the non-cirrhotic TBP group was significantly greater than that in the cirrhotic TBP group ($P < 0.05$).

The demographic and ascites data of the cirrhotic TBP group and the cirrhosis control group were compared (Table 2), and there were no significant differences in demographic data between the two groups. However, the mean values of ADA, total protein, WBC counts and lymphocyte counts in ascites of the cirrhotic TBP group were significantly higher than those of the cirrhosis control group ($P < 0.05$).

The distribution of ascites ADA values among the patient groups were compared (Figure 1), and the values of ascites ADA in the cirrhosis control group were markedly different from those of the other two groups with no overlapping area. Using 27 U/L as a cut-off value of ADA in ascites (the lowest value in the TBP group), no patient in the cirrhosis control group was found to have an ascites ADA concentration higher than this value.

The validation group included 217 patients, and subgroup analysis was performed according to the different etiologies of ascites (Figure 2). Using 27 U/L as a cut-off value of ascites ADA in the validation group, the sensitivity in detecting TBP was 100% and specificity was 93.3%. There were fifteen (6.9%) patients (one hepatocellular carcinoma, four malignancy other than hepatoma, five intra-abdominal infection, three nephrogenic ascites, one cardiogenic ascites and one hepatocellular carcinoma mixed with spontaneous bacterial peritonitis) who had ascites ADA levels higher than 27 U/L but did not have TBP.

Spearman's correlation analysis revealed that ascites ADA levels of all patients were strongly correlated with ascites total protein amounts ($r = 0.909$, $P < 0.001$) (Figure 3).

DISCUSSION

The utility of ascites ADA in the differential diagnosis of TBP for patients with underlying liver cirrhosis remains controversial due to conflicting results in previous studies. This is the first study to investigate cirrhotic patients with TBP involving a prospectively-enrolled cirrhosis control group, which was included to avoid the potential confounding effects of heterogeneous clinical conditions. In this study, even though lower levels of ascites ADA were found in the cirrhotic TBP group, their mean ascites ADA value was not significantly different from that of the non-cirrhotic TBP group. Moreover, ascites ADA values of cirrhotic patients with TBP were notably higher than those of the cirrhosis control group, and every pa-

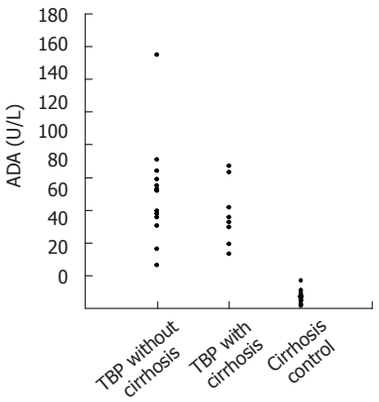


Figure 1 Adenosine deaminase distribution in the study groups. ADA: Adenosine deaminase; TBP: Tuberculous peritonitis.

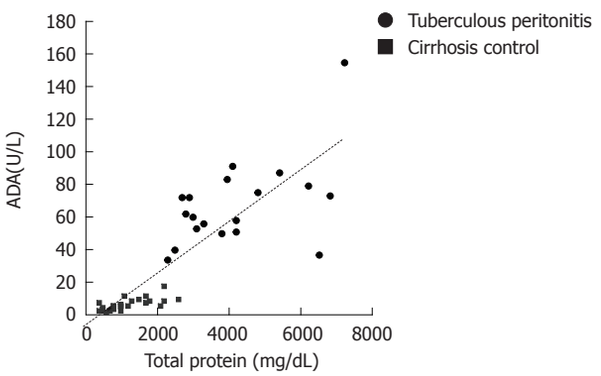


Figure 3 Correlation between adenosine deaminase and total protein in ascites among patients with tuberculous peritonitis. ADA: Adenosine deaminase.

tient in the cirrhosis control group had an ascites ADA level lower than the lowest value in the TBP group (27 U/L). Therefore, these data provide convincing evidence that ascites ADA may be significantly raised in TBP patients with underlying cirrhosis, and thus TBP should be considered in the differential diagnosis.

Previous studies showed high sensitivity and specificity in detecting TBP by checking ADA activity in ascites, based on ADA cut-off values of 36-40 IU/L[2,10,11,18]. However, Hillebrand *et al*[3] used only 7 IU/L as the cut-off value of ADA, and they found the sensitivity of ADA was only 30% in the setting of cirrhosis. There was considerable overlap in the ADA activity between TBP and sterile ascites among cirrhotic patients. Furthermore, Hillebrand *et al*[3] postulated that lower sensitivity and cut-off value might be caused by the higher proportion of cirrhosis (59%) in their patients with TBP. In contrast, Burgess *et al*[4] reported a sensitivity of 94% and a specificity of 92% for cirrhotic patients with TBP using a cut-off value for ADA of 30U/L. In the current study, there was no overlapping phenomenon in the ADA activity between TBP and sterile ascites among cirrhotic patients, and the sensitivity and specificity in detecting TBP were also high (100% and 93.3%, respectively) in the validation group using a cut-off value for ADA of 27 U/L. In

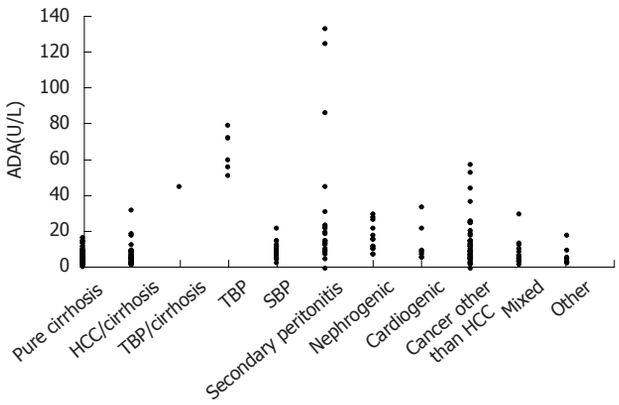


Figure 2 Adenosine deaminase distribution for various ascites etiologies in the validation group. ADA: Adenosine deaminase; TBP: Tuberculous peritonitis; SBP: Spontaneous bacterial peritonitis; HCC: Hepatocellular carcinoma.

addition, Hillebrand *et al*[3] determined ADA activity by detecting the decrease in adenosine concentration under the action of ADA, but this method was different from the measurement techniques applied in most previous studies. The methods employed by Slaats *et al*[17] or Giusti *et al*[19] have been extensively described in the literature[2,4,9,18], and involve determination of ADA activity by changes of NH_3 or NADH, respectively, after interacting ADA with adenosine. The different techniques used might affect the sensitivity and specificity of the ADA test, so the correlation of ADA values obtained from different methods need further investigation.

As shown in our validation group and in previous studies[2-4], false positive findings of ascites ADA are still possible. Peritoneal carcinomatosis and secondary bacterial peritonitis are the two most likely etiologies after TBP. ADA plays an important role in regulating the level of adenosine, and its primary function in humans is development and maintenance of the immune system[20,21]. ADA is involved in proliferation and differentiation of T lymphocytes, and diseases such as malignant conditions, collagen vascular diseases and some microorganism infections that are associated with lymphocytosis may increase ADA levels[22]. Therefore, for patients with elevated ascites ADA, further differential diagnosis by ascites cytological examination or radiological imaging studies should be performed, and laparoscopic visualization and biopsy may be considered for equivocal cases. Furthermore, due to the high negative predictive value (100%) found in this study, invasive procedures such as laparoscopic peritoneum biopsy or laparotomy, which are relatively high risk in cirrhosis patients, may be unnecessary and should be avoided when ascites ADA activity is low. In addition, the mean time to develop positive tuberculosis culture from ascites was 36.4 ± 18.2 d in our study, but the mean time in previous studies on ascites ADA was only 3.0 ± 0.5 d. Testing for ADA in ascites has high sensitivity and specificity, and may therefore be useful as a rapid test for diagnosis. Although TB polymerase chain reaction (PCR) has been used as a rapid diagnostic tool for pulmonary TB, PCR cannot be suggested for diagnosing TBP due to its

low sensitivity rate[9,23]. In endemic area of tuberculosis, DNA from dead TB bacilli may also give a false-positive result[9]. In addition, testing for ADA may also be currently more widely available than other valuable clinical tests such as interferon-gamma[24].

In this study, ADA activity was found to be strongly correlated with total protein in ascites in the TBP and control groups, and this finding was compatible with the simultaneously elevated total protein and ADA found in cirrhotic patients suffering from TBP. It is interesting to note that Fernandez-Rodriguez *et al*[15] found ADA was correlated with total protein in ascites among patients with TBP ($r = 0.842$), but the correlation was non-significant when the control group was included in the analysis. This inconsistent finding might be explained by heterogeneous etiologies and complex clinical conditions in the control group. Elevated total protein in the body fluid has been one of the diagnostic markers for inflammatory exudate[25], and it is also an important finding in ascites of TBP[5,15]. ADA is related to activation and differentiation of mononuclear leukocytes, and it is secreted during immune responses[3,21,26]. Lymphocytes are the predominant cells in ascites of TBP, and simultaneously elevated total protein and ADA can be explained by lymphocytic inflammation. Moreover, although mean total protein in the cirrhotic TBP group was significantly lower than that of the non-cirrhotic TBP group in this study, mean ADA levels were not significantly different. Compared with the cirrhosis control group, the WBC and lymphocyte counts were significantly higher in the cirrhotic TBP group, and the immune responses were predominant. Even in cirrhotic patients with a relatively immunocompromised status, TBP still activated strong immune reactions, which resulted in a sharp elevation of ADA in ascites, and this phenomenon indicates that ascites ADA may still be a valuable tool in the differential diagnosis of TBP.

We acknowledge several limitations in this study. Firstly, this study reflects the experience of a single medical center with a relatively small sample size, but the statistical power of the analysis was adequate. Secondly, cases with TBP were obtained from a computerized database search and retrospectively analyzed, so some TBP cases may have been missed due to incorrect coding in this long-term cohort study. Thirdly, we compared a historical TBP cohort with a prospectively-enrolled cirrhosis control group, and different time periods of study might produce some bias. However, patients were consecutively recruited, and the modalities for diagnosing TBP were not changed during the study period. The possible bias should be minimized.

In conclusion, even with lower ascites ADA activity in liver cirrhosis, ascites ADA levels could be significantly elevated due to strong immune responses when cirrhotic patients suffer from TBP. Owing to its high sensitivity and specificity, ascites ADA may be a valuable tool in the differential diagnosis of TBP in patients with underlying cirrhosis, and concomitant cirrhosis should not limit its clinical utility.

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COMMENTS

Background

The risk of tuberculous peritonitis (TBP) is increased in patients with underlying liver cirrhosis. Adenosine deaminase (ADA) in ascites has been proposed to be a useful test for early detection of TBP, but its value among patients with underlying cirrhosis is uncertain.

Research frontiers

The utility of ascites ADA in the differential diagnosis of TBP in patients with underlying liver cirrhosis remains controversial due to conflicting results in previous studies. In addition, the relationship between ascites ADA and other parameters such as total protein has not been well discussed, and the mechanisms of ADA elevation among cirrhotic patients with TBP need further investigation.

Innovations and breakthroughs

This is the first study to investigate cirrhotic patients with TBP which includes a prospectively-enrolled cirrhosis control group, and ADA activity was strongly correlated with total protein in ascites. Even with lower ascites ADA activity in liver cirrhosis, ascites ADA levels could be significantly elevated due to strong immune responses when cirrhotic patients suffer from TBP.

Applications

Owing to its high sensitivity and specificity, ascites ADA may be a valuable tool in the differential diagnosis of TBP in patients with underlying cirrhosis, and concomitant cirrhosis should not limit its clinical utility. Furthermore, due to the high negative predictive value, invasive procedures such as laparoscopic peritoneum biopsy or laparotomy may be unnecessary when ascites ADA activity is low.

Terminology

ADA is an enzyme involved in purine metabolism, and it can be a product of immune responses relating to T lymphocyte activity in humans.

Peer review

This is a good case-control study and the message is clear. The authors examined ADA activity in TBP among patients with underlying liver cirrhosis, and a prospectively-enrolled cirrhosis control group was conducted to avoid the potential confounding effects of heterogeneous clinical conditions. Due to convincing data in this study, ascites ADA can still be a valuable tool in the differential diagnosis of TBP.

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BRIEF ARTICLE

Volumetric-modulated arc therapy *vs* c-IMRT in esophageal cancer: A treatment planning comparison

Li Yin, Hao Wu, Jian Gong, Jian-Hao Geng, Fan Jiang, An-Hui Shi, Rong Yu, Yong-Heng Li, Shu-Kui Han, Bo Xu, Guang-Ying Zhu

Li Yin, Hao Wu, Jian Gong, Jian-Hao Geng, Fan Jiang, An-Hui Shi, Rong Yu, Yong-Heng Li, Shu-Kui Han, Bo Xu, Guang-Ying Zhu, Department of Radiotherapy, Peking University School of Oncology, Beijing Cancer Hospital and Institute, Beijing 100142, China

Author contributions: Yin L took charge of the whole work, carried out data collection, performed treatment planning and drafted the manuscript; Wu H, Gong J, Jiang F, Han SK and Xu B performed physics checkups and plan evaluation; Shi AH, Yu R and Li YH determined the patient accrual and radiation field; Zhu GY, Xu B and Han SK provided mentorship for this work; Wu H and Geng JH revised the manuscript; Zhu GY designed the study and approved the final version to be published; and all authors have read and approved the final manuscript.

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Correspondence to: Guang-Ying Zhu, MD, PhD, Department of Radiotherapy, Peking University School of Oncology, Beijing Cancer Hospital and Institute, Beijing 100142, China. zgypu@yahoo.com.cn
Telephone: +86-10-88196120 Fax: +86-10-88196120

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Abstract

AIM: To compare the volumetric-modulated arc therapy (VMAT) plans with conventional sliding window intensity-modulated radiotherapy (c-IMRT) plans in esophageal cancer (EC).

METHODS: Twenty patients with EC were selected, including 5 cases located in the cervical, the upper, the middle and the lower thorax, respectively. Five plans were generated with the eclipse planning system: three using c-IMRT with 5 fields (5F), 7 fields (7F) and 9 fields (9F), and two using VMAT with a single arc (1A) and double arcs (2A). The treatment plans were designed to deliver a dose of 60 Gy to the planning target volume (PTV) with the same constrains in a 2.0 Gy daily fraction, 5 d a week. Plans were normalized to 95% of the PTV that received 100% of the prescribed dose. We examined the dose-volume histogram parameters of PTV and the organs at risk (OAR) such as lungs, spinal cord and heart. Monitor units (MU) and normal tissue complication probability (NTCP) of OAR were also reported.

RESULTS: Both c-IMRT and VMAT plans resulted in abundant dose coverage of PTV for EC of different locations. The dose conformity to PTV was improved as the number of field in c-IMRT or rotating arc in VMAT was increased. The doses to PTV and OAR in VMAT plans were not statistically different in comparison with c-IMRT plans, with the following exceptions: in cervical and upper thoracic EC, the conformity index (CI) was higher in VMAT (1A 0.78 and 2A 0.8) than in c-IMRT (5F 0.62, 7F 0.66 and 9F 0.73) and homogeneity was slightly better in c-IMRT (7F 1.09 and 9F 1.07) than in VMAT (1A 1.1 and 2A 1.09). Lung V30 was lower in VMAT (1A 12.52 and 2A 12.29) than in c-IMRT (7F 14.35 and 9F 14.81). The humeral head doses were significantly increased in VMAT as against c-IMRT. In the middle and lower thoracic EC, CI in VMAT (1A 0.76 and 2A 0.74) was higher than in c-IMRT (5F 0.63 Gy and 7F 0.67 Gy), and homogeneity was almost similar between VMAT and c-IMRT. V20 (2A 21.49 Gy *vs* 7F 24.59 Gy and 9F 24.16 Gy) and V30 (2A 9.73 Gy *vs* 5F 12.61 Gy, 7F 11.5 Gy and 9F 11.37 Gy) of lungs in VMAT were lower than in c-IMRT, but low doses to lungs (V5 and V10) were increased. V30 (1A 48.12 Gy *vs* 5F 59.2 Gy, 7F 58.59 Gy and 9F 57.2 Gy), V40 and V50 of heart in VMAT was lower than in c-IMRT. MUs in VMAT plans were significantly reduced in comparison with c-IMRT, maximum doses to the spinal cord and mean doses of lungs were similar between the two techniques. NTCP of spinal cord was 0 for all cases. NTCP of lungs and heart in VMAT were lower than in

c-IMRT. The advantage of VMAT plan was enhanced by doubling the arc.

CONCLUSION: Compared with c-IMRT, VMAT, especially the 2A, slightly improves the OAR dose sparing, such as lungs and heart, and reduces NTCP and MU with a better PTV coverage.

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Key words: Esophageal cancer; Treatment planning; Intensity modulated radiotherapy; Volumetric modulated arc radiotherapy; Normal tissue complication probability

Peer reviewer: Stéphane Supiot, MD, PhD, Department of Radiation Oncology, Centre René Gauducheau, St-Herblain, 44800 Nantes, France

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INTRODUCTION

Esophageal cancer (EC) is one of the most common malignancies in the world. It was estimated that there were 16 470 newly diagnosed cases of EC, and 14 280 cases of death in America in 2008[1]. Squamous cell carcinoma is commonly seen in China, whereas adenocarcinoma is common in Europe and America. Radiotherapy is a major treatment method for EC because more than 60% of the patients are often diagnosed at locally advanced stages which could not be totally resected. Innovative technologies in radiation delivery such as intensity-modulated radiotherapy (IMRT) offer the potential for improved tumor coverage, while reducing the doses delivered to the surrounding normal tissues. Clinical studies have yielded good dosimetry and patient outcome by IMRT[2-6]. There are different IMRT delivery approaches, including "step and shoot", "sliding window" modes and the rotational technique. Volumetric-modulated arc therapy (VMAT), the novel form of IMRT that was first proposed by Yu in 1995[7], allowed for intensity-modulated radiation delivery during gantry rotation with dynamic multi-leaf collimator (MLC) motion, variable dose rates (DR) and gantry speed modulation. VMAT had already been investigated for prostate cancer, small brain tumors and cervix uteri cancer[8-10]. These studies have generally shown that VMAT is able to produce similar or better dose distributions, while achieving a reduction in treatment time and a reduction in monitor units (MU).

We performed a planning study to compare VMAT with conventional sliding window intensity-IMRT (c-IMRT) in EC of all locations and in dose distributions to planning target volume (PTV) and organs at risk (OAR). We also investigated the difference of normal tissue complication probability (NTCP) between the two techniques.

MATERIALS AND METHODS

Patients

Twenty EC patients treated with c-IMRT previously in our department were selected for this study, involving 5 cases of EC located in the cervical, the upper, the middle and the lower thorax, respectively. Five patients were staged Ⅱ, 10 were Ⅲ and 5 were Ⅳ according to the American Joint Committee (AJCC) on Cancer 2006 Guidelines. Details are shown in Table 1.

Target volume and organ at risk delineation

All patients were immobilized in a supine position and computed tomography scanned using a helical scanner (Siemens Somatom, Sensation Open Computed Tomography) with 1.25 mm thick slices over the neck and the entire thorax. The clinical target volume, including the esophageal tumor, with a margin for microscopic tumor extension, and the adjacent regional lymph nodes[11,12], was expanded with a 5-mm margin to create PTV. OAR, such as spinal cord, heart and lung, was outlined on each image. Details of the delineation of these volumes were recently described[13].

Planning techniques and objectives

All the treatment plans were designed to deliver 60 Gy to the PTV in 30 fractions using the Eclipse treatment planning system (Version 8.9, Varian Medical Systems, Palo Alto, CA), with 6 MV photon beam from a Varian Ⅸ (RapidArc) equipped with a Millennium MLC with 120 leaves. The Anisotropic Analytical Algorithm (Version 8.9) photon dose calculation algorithm and dose calculation grid of 2.5 mm were used for both c-IMRT and VMAT. When necessary, field size was minimized to 15.3 cm in the X direction. This dimension corresponded of the maximal displacement of a leaf in a MLC bank. By doing so, all the leave positions were possible during the optimization process increasing the degree of modulation even if in a beam eye view, a part of the volume was excluded of the beam at each gantry position. Globally rotational delivery permitted to irradiate all the volume of the PTV during rotation. All plans aimed to achieve a minimum dose larger than 95% and a maximum dose lower than 107% of the prescribed dose, and no 2-cc region (either in or outside of PTV) may receive > 110% of the dose. With regard to the OAR, the primary objectives were defined as follows: spinal cord: D_{max} < 45 Gy and lungs: V20 < 30%. The secondary objectives were: mean doses of lungs (MLD) < 15 Gy and heart: V40 ⩽ 50%, V50 ⩽ 40%. As a result of tumor coverage requirements, a waiver can be applied on these dose constraints.

VMAT plans

The VMAT plans using full arcs sharing the same isocenter, in which 1A consisting of a single 360° rotation

Table 1 Characteristics of patients (n = 20)

Variables	n
Gender	
Male	16
Female	4
Age range (yr)	45-82
Stage[1]	
Ⅱ	5
Ⅲ	10
Ⅳ	5
Histology	
Squamous carcinoma	18
Adenocarcinoma	2

[1]According to the American Joint Committee on Cancer 2006 Guidelines.

and 2A consisting of two coplanar arcs of 360° with opposite rotation (clock-wise or counter clock-wise), were optimized selecting a maximum DR of 600 MU/min. For 1A, starting at a gantry angle of 179° and rotating counter clockwise at 360° to stop at a gantry angle of 181°, field size and collimator rotation were determined by the automatic tool from Eclipse to encompass the PTV. And 2A, consisting of two coplanar arcs of 360°, was optimized simultaneously with opposite rotation. Since each individual arc is limited to a sequence of 177 control points, the application of two coplanar arcs that increase the modulation factor during optimization, could allow the optimizer to achieve a higher target homogeneity and lower OARs involvement at the same time. For the second arc, the collimator was rotated 5° extra to reduce overlapping tongue and groove effects with the first arc. Details about VMAT optimization process have been published elsewhere[14].

c-IMRT plans

The c-IMRT plans were optimized with a fixed DR of 400 MU/min. The MLC leaf sequences were generated using the dynamic sliding window IMRT delivery technique. Plans were individually optimized using five (5F), seven (7F) and nine (9F) coplanar fields. Beam geometry consisted of each treatment field with the following gantry angles: 0°/50°/153°/204°/310° (5F), 20°/60°/150°/180°/210°/300°/340° (7F), and 0°/35°/70°/130°/160°/200°/230°/290°/325° (9F).

Once the treatment planning was completed, the plan was normalized to cover 95% of the PTV with 100% of the prescribed dose. In the present study, we tried to modify constraints and priority factors in the c-IMRT and VMAT plans to improve the results. These parameters were modified in function of dose-volume histogram (DVH) results for each patient.

Evaluation tools

Analysis was performed on DVH computing several standard parameters[15], Dx was the specific dose computed for a fraction of a target or an organ volume, and Vx was the volume irradiated above a designated dose. For PTV, the mean dose (D_{mean}) was analyzed, and the conformity of dose distribution was assessed by means of conformity index (CI) which was defined as the ratio between the volume receiving at least 95% of 60 Gy and the volume of the PTV. Higher values of CI represented a better PTV conformity. CI = (VT95%/VT) × (VT95%/V95%)[16].

The homogeneity index (HI) of the PTV was computed as D5%-D95% (difference between the dose covering 5% and 95% of the PTV). Lower values of HI represented a more homogenous PTV dose distribution[17].

DVH parameters for OARs (spinal cord, lungs and heart) were calculated and compared. A set of Vx values, D_{mean}, D_{max} and MU was therefore reported.

Radiobiological comparison was analyzed by the NTCP. The risk of developing acute complications and other late complications was assessed using the Lyman-Kutcher-Burman model[18]. The parameters for NTCP calculations (volume effect, slope, and tolerance doses) were taken from Burman *et al*[19] and are shown in Table 2.

Statistical analysis

The Wilcoxon matched-pair signed-rank test was used to compare the results between the VMAT and IMRT plans. Difference was considered statistically significant at $P < 0.05$. All statistical tests were two-sided, and all statistical analyses were done using the SPSS software, Version 11.0 (Chicago, IL).

RESULTS

Target coverage, conformity and dose homogeneity

Clinically acceptable plans of VMAT and c-IMRT were completed by all the 20 patients. The dosimetric results of each position for PTV are listed in Table 3. The results were analogous in the cervical and upper EC, for which PTV was T-shaped from a posteroanterior view, while PTV was I-shaped in middle and lower EC. As the numbers of field in c-IMRT or arc in VMAT were increased, the conformity and homogeneity were improved.

For D_{mean} of PTV, VMAT (1A and 2A) yielded higher values than IMRT (5F, 7F and 9F). There was significant difference between VMAT and c-IMRT (7F and 9F) in cervical and upper thoracic EC and c-IMRT (5F and 7F) in the middle and lower thoracic EC, and only 1A achieved a higher D_{mean} as compared with IMRT ($P < 0.05$).

VMAT had a better CI than c-IMRT. Statistically significant difference was seen between VMAT and c-IMRT (5F, 7F and 9F) in cervical and upper thoracic EC, but between VMAT and c-IMRT (5F and 7F) in middle and lower thoracic EC ($P < 0.05$). Especially in cervical cases, 2A showed the best CI ($P < 0.05$), but there was no significant difference between 1A and 2A in thoracic cases.

Homogeneity was slightly better in c-IMRT than in VMAT. In cervical and upper thoracic EC, HI of 2A and 5F was equivalent, and 7F or 9F showed a significant trend for better results compared with VMAT ($P < 0.05$). In the middle and lower thoracic EC, the trend was not conspicuous, 9F also had a higher HI compared with

Table 2 Parameters used in normal tissue complication probability

Organ	Size factor (*n*)	Slope (m)	TD5/5 (Gy)	TD50/5 (Gy)	End point
Lung	0.87	0.18	17.5	24.5	Pneumonitis
Heart	0.35	0.10	40	48	Pericarditis
Spinal cord	0.05	0.175	47	66.5	Myelitis/necrosis

TD5/5: Tolerance dose leading to 5% complication rates at 5 years; TD50/5: Tolerance dose leading to 50% complication rates at 5 years.

Table 3 Dosimetric results for planning target volume and monitor units

Variable	IMRT-5F	IMRT-7F	IMRT-9F	VMAT-1A	VMAT-2A	$P < 0.05$
D_{mean}						
Cervical	63.68 ± 0.37	63.07 ± 0.36	62.55 ± 0.39	63.97 ± 0.08	63.63 ± 0.49	2A *vs* 7F and 9F
Upper	63.30 ± 0.66	62.74 ± 0.49	62.38 ± 0.34	63.90 ± 0.45	63.43 ± 0.63	1A, 2A *vs* 7F and 9F
Middle	64.12 ± 1.03	64.05 ± 1.27	63.88 ± 1.27	64.83 ± 1.06	64.21 ± 0.59	1A *vs* 5F and 9F
Lower	63.14 ± 0.90	63.20 ± 1.09	62.98 ± 0.87	64.17 ± 1.26	63.98 ± 1.36	1A *vs* 5F, 7F and 9F
HI						
Cervical	1.10 ± 0.01	1.09 ± 0.01	1.07 ± 0.01	1.11 ± 0.00	1.10 ± 0.01	1A, 2A *vs* 7F and 9F
Upper	1.09 ± 0.02	1.08 ± 0.01	1.07 ± 0.01	1.10 ± 0.01	1.09 ± 0.02	1A *vs* 7F and 9F; 2A *vs* 9F
Middle	1.11 ± 0.02	1.11 ± 0.03	1.11 ± 0.03	1.12 ± 0.02	1.11 ± 0.01	1A *vs* 9F
Lower	1.09 ± 0.02	1.09 ± 0.03	1.09 ± 0.02	1.11 ± 0.04	1.11 ± 0.03	1A *vs* 9F
CI						
Cervical	0.63 ± 0.03	0.66 ± 0.02	0.74 ± 0.04	0.78 ± 0.03	0.80 ± 0.03	2A *vs* 5F, 7F, 9F and 1A
Upper	0.62 ± 0.04	0.66 ± 0.03	0.73 ± 0.02	0.79 ± 0.03	0.80 ± 0.02	1A, 2A *vs* 5F, 7F and 9F
Middle	0.62 ± 0.09	0.67 ± 0.08	0.71 ± 0.10	0.76 ± 0.05	0.74 ± 0.08	1A, 2A *vs* 5F and 7F
Lower	0.64 ± 0.05	0.67 ± 0.05	0.71 ± 0.05	0.76 ± 0.05	0.77 ± 0.04	1A, 2A *vs* 5F and 7F
MU						
Cervical	1088 (921-1157)	1261 (1094-1393)	1236 (1004-1413)	610 (546 -665)	525 (452-590)	2A *vs* 5F, 7F, 9F and 1A
Upper	1110 (841-1244)	1251 (950-1377)	1334 (1040-1592)	679 (538-825)	682 (475-1004)	1A, 2A *vs* 5F, 7F and 9F
Middle	831 (707-980)	903 (808-1086)	999 (858-1219)	418 (347-459)	431 (376-503)	1A, 2A *vs* 5F, 7F and 9F
Lower	826 (721-966)	923 (720-1234)	1086 (958-1375)	440 (387-540)	419 (347-531)	1A , 2A *vs* 5F, 7F and 9F

IMRT: Intensity modulated radiotherapy; VMAT: Volumetric modulated arc therapy; F: Coplanar field; A: Arc; HI: Homogeneity index; CI: Conformity index; D_{mean}: Mean dose; MU: Monitor units.

other plans, and significant difference was found only in 9F and 1A ($P < 0.05$). Figure 1 depicts the dose distribution of c-IMRT and VMAT in a cervical EC patient.

OAR

The absolute plan parameters for lungs, heart and spinal cord are summarized in Table 4. DVH of OAR in one patient were shown in Figure 2.

The reduction trend of lung parameters (V5, V10, V20 and V30) was similar between the two techniques, except for MLD. In cervical and upper thoracic EC, MLD, V20 and V30 by VMAT were reduced by 0.6%-2.9%, 2.1%-10.7% and 13.2%-17.3%, respectively. V5 and V10 of lung by VMAT in cervical and upper thoracic EC were increased by 5.5%-7.7% and 10.5%-12.6%, respectively. In middle and lower thoracic EC, VMAT resulted in increased V5 (10.6%-13.3%), V10 (18.4%-21.8%) and MLD (2%-2.3%), but decreased V20 (5.5%-15.5%) and V30 (13.2%-18.2%). Statistically significant difference was found between VMAT (1A or 2A) and c-IMRT (5F, 7F and 9F) for V5, V10, V20 and V30, but for MLD, there was significant difference between VMAT (1A or 2A) and c-IMRT (5F, 7F) ($P < 0.05$).

For the heart, VMAT reduced V30, V40 and V50 as compared with c-IMRT, especially in thoracic cases. V30 by VMAT was reduced by 33.5%, 10.7% and 21.6%, V40 by 36%, 15.1% and 21.7%, and V50 by 39.3%, 29.7% and 38% in the upper, middle and lower thoracic EC, respectively. VMAT (1A or 2A) reduced V30 and V40 (5F, 7F and 9F) in thoracic EC, and V50 in middle and lower thoracic EC ($P < 0.05$). However, no difference was found in the D_{max} of spinal cord between VMAT and c-IMRT.

NTCP results are shown in Table 5. VMAT (1A or 2A) significantly lowered the NTCP in comparison with c-IMRT (5F, 7F and 9F) in cervical and upper thoracic cases, while there was significant difference between 2A and 5F in middle and lower thoracic cases ($P < 0.05$). The trend of cardiac NTCP in VMAT was similar with lungs in thoracic EC, especially in middle and lower thoracic EC ($P < 0.05$).

It was worth noting that D_{mean} and maximal doses to the humeral head (HH_{mean} and HH_{max}) in VMAT were dramatically increased in comparison with c-IMRT in cervical and upper thoracic EC (Table 4). Compared with c-IMRT, HH_{mean} in VMAT was increased by almost three times in cervical EC and four times in upper thoracic EC ($P < 0.05$). HH_{max} in VMAT was twice higher in cervical EC and three times higher in upper thoracic EC than that in c-IMRT (5F and 7F) ($P < 0.05$).

Table 4 Dosimetric comparison for organs at risk of conventional sliding window intensity-modulated radiotherapy and volumetric-modulated arc therapy in cervical and upper thoracic esophageal cancer, and in middle and lower thoracic esophageal cancer, mean value (range)

Organ	Variable	c-IMRT (5F, 7F, 9F)	VMAT (1A, 2A)	Relative reduction (%)	$P < 0.05$
In cervical and upper thoracic					
Lung (Gy)	MLD[1]	12.65 (12.38-13.04)	12.57 (12.35-12.79)	0.6	2A *vs* 7F, 9F; 1A *vs* 7F
	MLD[2]	14.35 (13.91-14.76)	13.94 (13.74-14.14)	2.9	2A *vs* 7F, 9F
	V5[1]	48.52 (46.28-50.52)	51.20 (51.03-51.37)	-5.5	1A, 2A *vs* 5F, 7F
	V5[2]	61.53 (57.51-65.06)	66.25 (66.07-66.43)	-7.7	1A , 2A *vs* 5F, 7F
	V10[1]	39.30 (37.41-41.58)	43.44 (43.23-43.65)	-10.5	1A , 2A *vs* 5F, 7F; 2A *vs* 9F
	V10[2]	48.17 (44.18-52.07)	54.25 (53.70-54.79)	-12.6	1A , 2A *vs* 5F, 7F, 9F
	V20[1]	24.70 (23.93-25.16)	24.17 (23.08-25.26)	2.1	1A *vs* 5F
	V20[2]	25.58 (24.29-26.23)	22.85 (21.94-23.76)	10.7	NS
	V30[1]	14.96 (14.42-15.33)	12.99 (12.99-12.99)	13.2	1A, 2A *vs* 5F, 7F, 9F
	V30[2]	14.52 (14.27-15.00)	12.01 (11.58-12.04)	17.3	1A, 2A *vs* 5F, 7F; 2A *vs* 9F
Heart (%)	V30[1]	6.83 (5.7-7.41)	5.30 (4.57-5.62)	22.4	NS
	V30[2]	15.20 (13.62-16.96)	10.11 (9.98-10.24)	33.5	1A *vs* 7F, 9F, 2A
	V40[1]	4.33 (3.55-5.08)	2.68 (2.45-2.91)	38.1	NS
	V40[2]	8.32 (7.00-10.33)	5.30 (5.08-5.22)	36	1A *vs* 5F, 7F, 9F; 2A *vs* 5F
	V50[1]	2.62 (2.07-3.09)	1.44 (1.43-1.45)	45	NS
	V50[2]	4.33 (3.44-5.53)	2.63 (2.59-2.67)	39.3	NS
Spinal cord (Gy)	D_{max}[1]	37.92 (37.53-38.32)	37.74 (37.31-38.17)	0.5	NS
	D_{max}[2]	37.85 (37.41-38.43)	38.41 (38.05-38.76)	-1.5	NS
Head of humerus	D_{max}[1]	10.00 (5.97-17.59)	21.88 (20.80-22.95)	-118.8	1A , 2A *vs* 5F, 7F, 9F
(Gy)	D_{max}[2]	7.57 (6.47-9.61)	26.44 (26.35-26.52)	-249.3	1A , 2A *vs* 5F, 7F
	D_{mean}[1]	3.24 (2.07-4.71)	12.27 (11.85-12.69)	-278.7	2A *vs* 5F, 7F, 9F, 1A
	D_{mean}[2]	2.89 (1.47-4.86)	15.26 (14.61-15.90)	-428	2A *vs* 5F, 7F, 9F, 1A
In middle and lower thoracic					
Lung (Gy)	MLD[3]	15.03 (14.86-15.27)	15.38 (15.24-15.51)	-2.3	1A, 2A *vs* 5F, 7F
	MLD[4]	15.37 (15.01-15.81)	15.67 (15.51-15.82)	-2	1A *vs* 5F
	V5[3]	74.86 (71.00-79.05)	82.83 (82.72-82.93)	-10.6	1A, 2A *vs* 5F, 7F, 9F
	V5[4]	79.36 (72.43-85.85)	89.91 (89.77-90.04)	-13.3	1A, 2A *vs* 5F, 7F, 9F
	V10[3]	55.14 (52.64-58.66)	65.26 (64.75-65.76)	-18.4	1A, 2A *vs* 5F, 7F, 9F
	V10[4]	59.56 (54.27-64.76)	72.54 (72.04-73.03)	-21.8	1A, 2A *vs* 5F, 7F; 2A *vs* 9F
	V20[3]	24.39 (23.84-24.96)	23.05 (22.28-23.81)	5.5	2A *vs* 7F, 9F, 1A
	V20[4]	25.32 (24.48-26.66)	21.40 (20.69-22.11)	15.5	1A, 2A *vs* 5F
	V30[3]	12.64 (12.08-13.01)	10.97 (10.88-11.06)	13.2	1A,2A *vs* 5F, 7F; 2A *vs* 9F
	V30[4]	11.13 (10.55-12.20)	9.10 (8.58-9.62)	18.2	1A, 2A *vs* 5F, 7F; 2A *vs* 9F
Heart (%)	V30[3]	47.30 (46.61-48.20)	42.26 (40.72-43.79)	10.7	1A *vs* 5F, 7F, 9F
	V30[4]	72.16 (67.81-78.60)	56.55 (55.52-57.57)	21.6	1A *vs* 5F, 7F, 9F; 2A *vs* 5F
	V40[3]	26.89 (24.86-28.14)	22.84 (20.98-24.69)	15.1	1A *vs* 7F
	V40[4]	33.53 (30.63-38.19)	26.27 (26.21-26.32)	21.7	2A *vs* 5F
	V50[3]	14.06 (12.50-16.06)	9.89 (8.93-10.85)	29.7	1A *vs* 5F, 7F, 9F
	V50[4]	17.99 (14.48-22.87)	11.16 (10.93-11.39)	38	1A, 2A *vs* 5F; 2A *vs* 7F, 9F
Spinal cord (Gy)	D_{max}[3]	39.03 (38.91-39.15)	38.70 (38.54-38.86)	0.8	NS
	D_{max}[4]	37.61 (37.45-37.91)	37.99 (37.78-38.20)	-1	NS

Wilcoxon's signed ranks test. [1]Cervical thoracic esophageal cancer; [2]upper thoracic esophageal cancer; [3]middle thoracic esophageal cancer; [4]lower thoracic esophageal cancer. c-IMRT: Conventional sliding window intensity-modulated radiotherapy; VMAT: Volumetric-modulated arc therapy; MLD: Mean dose of lungs; D_{max}: Maximal dose; D_{mean}: Mean dose; F: Coplanar field; A: Arc; Vx: The percentage of organ receiving a dose > X Gy; NS: Not significant.

Monitor units

The c-IMRT plans required an increased MU per fraction when the field was increased whereas the VMAT plans usually resulted in lower MU when rotating arcs were increased. 1A plans required at least 50% or 60% less than 9F in cervical and upper EC or middle and lower EC ($P < 0.05$). The difference between VMAT (1A and 2A) and c-IMRT (5F, 7F and 9F) remained significant ($P < 0.05$) in all the cases. Detailed information about MU is shown in Table 3.

DISCUSSION

In the present study, VMAT proved to be slightly better than c-IMRT for targeting dose distribution in EC of all locations, and to have equivalent or better OAR dose sparing and lower NTCP. We initiated a dosimetric and radiobiological comparison in the EC of all locations in this study. The results indicated that VMAT could generate better radiotherapeutic plans than sliding window IMRT.

VMAT is a complex form of IMRT that allows dose delivery in single or multiple arcs. Two arcs allowed superior modulation factor during optimization due to the independent optimization, and unrelated sequence of MLC shape, gantry speed and dose rate combinations. This approach provided adequate coverage of PTV and spare of OARs at least equivalent to c-IMRT,

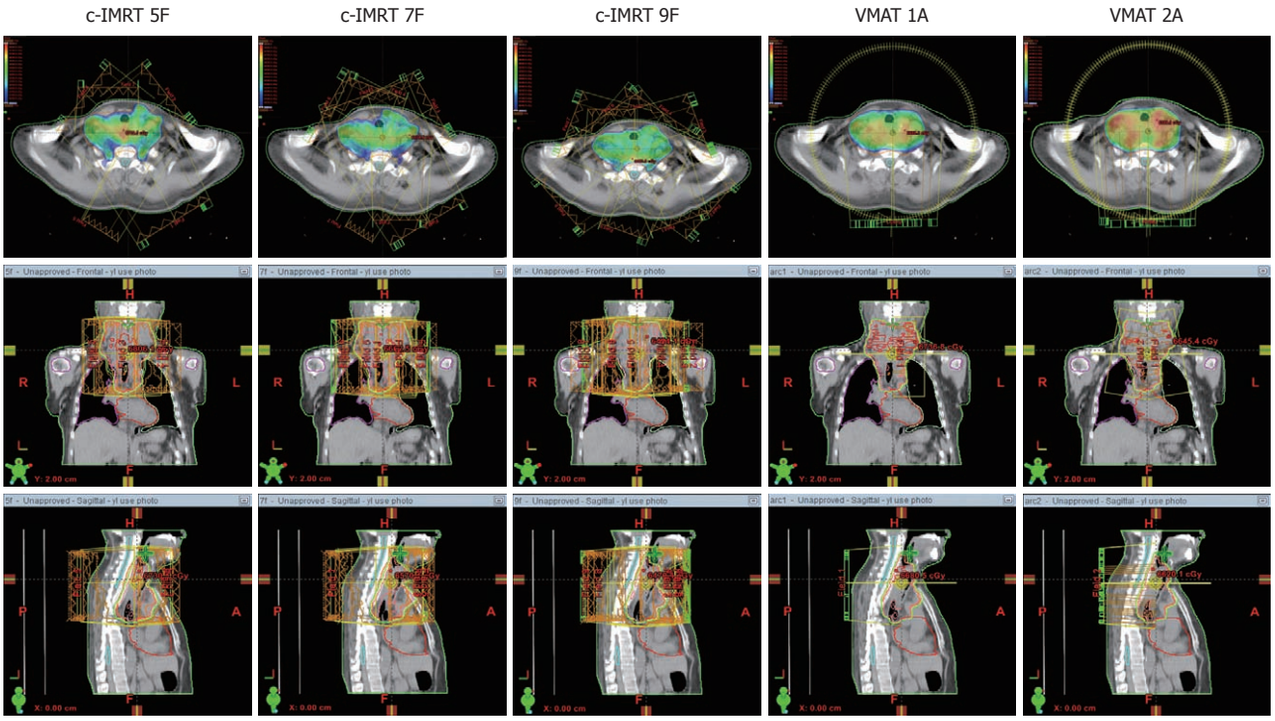


Figure 1 Dose distributions in a cervical esophageal cancer patient planed by conventional sliding window intensity-modulated radiotherapy (5 fields, 7 fields and 9 fields) and volumetric-modulated arc therapy (1 arc and 2 arcs). IMRT: Intensity-modulated radiotherapy; VMAT: Volumetric modulated arc therapy; F: Coplanar field; A: Arc; Orange line: Planning target volume; Blue line: Spinal cord; Color wash areas: Receiving ⩾ 100% of the dose (60 Gy).

Table 5 Normal tissue complication probability results for organs at risk

Organ	IMRT-5F	IMRT-7F	IMRT-9F	VMAT-1A	VMAT-2A	$P < 0.05$
Lung						
Cervical	0.24 ± 0.14	0.24 ± 0.15	0.32 ± 0.22	0.22 ± 0.18	0.13 ± 0.09	1A *vs* 9F; 2A *vs* 5F, 7F, 9F
Upper	0.73 ± 0.62	0.77 ± 0.66	0.84 ± 0.71	0.41 ± 0.37	0.30 ± 0.23	1A *vs* 7F, 9F; 2A *vs* 5F, 7F, 9F
Middle	0.62 ± 0.48	0.59 ± 0.48	0.60 ± 0.47	0.57 ± 0.45	0.50 ± 0.41	2A *vs* 5F
Lower	0.59 ± 0.45	0.59 ± 0.50	0.67 ± 0.46	0.57 ± 0.54	0.46 ± 0.37	2A *vs* 5F
Heart						
Cervical	0	0	0	0	0	NS
Upper	0.02 (0-0.12)	0.02 (0-0.09)	0 (0-0.02)	0	0	NS
Middle	0.61 (0.05-1.07)	0.94 (0.01-4.24)	0.31 (0.01-1.17)	0.13 (0-0.58)	0.21 (0-0.80)	1A *vs* 5F, 7F, 9F
Lower	6.66 (1.14-16.04)	1.97 (0.11-5.09)	1.76 (0.07-5.95)	1.32 (0-5.81)	0.66 (0-1.59)	1A *vs* 5F, 9F; 2A *vs* 5F, 7F, 9F
Spinal cord						
Cervical	0	0	0	0	0	NS
Upper	0	0	0	0	0	NS
Middle	0.002 (0-0.01)	0.006 (0-0.03)	0.008 (0-0.04)	0	0	NS
Lower	0	0	0	0	0	NS

IMRT: Intensity-modulated radiotherapy; VMAT: Volumetric modulated arc therapy; F: Coplanar field; A: Arc; NS: Not significant.

while it could reduce significantly the treatment time and the number of MU required in the morbidities, such as head and neck cancer, intracranial tumor, breast cancer, glioma, and carcinoma of the anal canal[14,17,20,21]. In the present study, VMAT using 2A achieved better results than using 1A.

First, the PTV volumes were larger in cervical and upper EC series due to pathological characteristics and biological behavior of carcinoma in these regions, and presented T-shaped from a posteroanterior view. In head and neck carcinoma, due to more complex target volume, 7F or 9F are constantly used in c-IMRT to meet the requirements of dose distribution, HI and CI of PTV[22,23]. Our results were consistent with this. PTV coverage in c-IMRT with 5F was less qualified in comparison with 7F or 9F. Both VMAT and c-IMRT resulted in abundant D_{mean} in PTV (63.94 Gy and 63.53 Gy in 1A and 2A *vs* 63.49 Gy, 62.90 Gy, 62.46 Gy in 5F, 7F and 9F). VMAT proved to be superior to c-IMRT in terms of MU and CI, but slightly inferior to c-IMRT in terms of HI. We also confirmed that VMAT with 2A achieved better results than 1A in terms of conformity and homogeneity. For the heart, VMAT showed a lower percentage of V30, V40 or V50. For lungs, VMAT provided

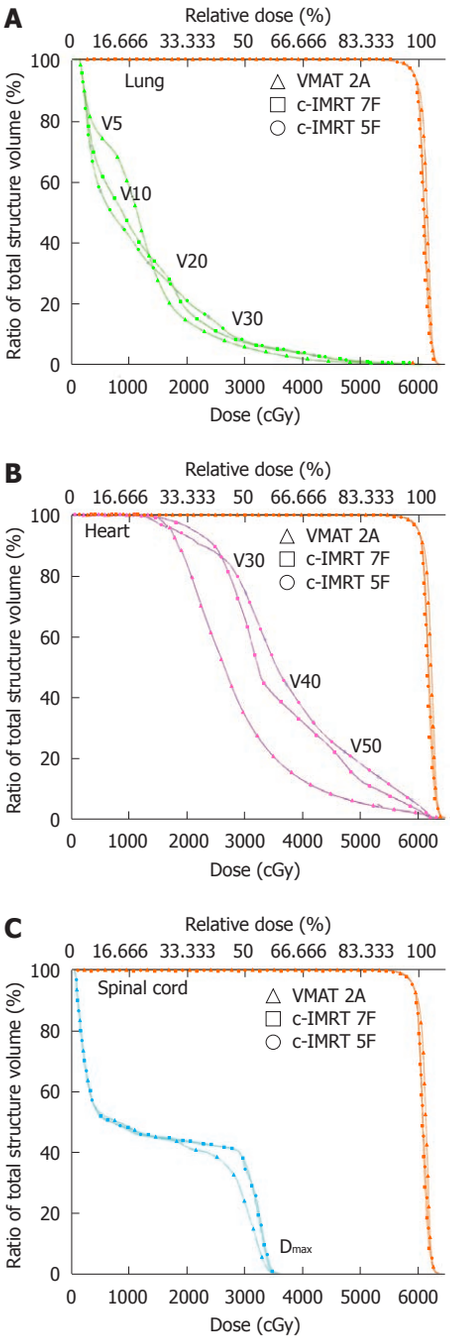


Figure 2 Dose-volume histogram of organs at risk and planning target volume for volumetric-modulated arc therapy and conventional sliding window intensity-modulated radiotherapy in a lower thoracic esophageal cancer patient. Volumetric-modulated arc therapy (VMAT) with double arcs (triangle) and conventional sliding window intensity-modulated radiotherapy (c-IMRT) with 7 fields (squares) and 5 fields (round). The planning target volume is shown in orange, the lungs in green (A), heart in red (B) and spinal cord in blue (C). F: Coplanar field; A: Arc; Vx: The percentage of organ receiving a dose > X Gy.

better sparing in terms of V20 and V30. The results of radiobiological NTCP comparison demonstrated that VMAT was superior to c-IMRT either in lungs or heart ($P < 0.05$). Yin *et al*[24] compared 7F in IMRT with VMAT plans for cervical EC, and found that there were differences between VMAT and IMRT in HI and MU, but not in CI, which are consistent with our results, but lung V5 in VMAT (1A 51.4, Ax 49.3 and 7F 50.9) was reduced while lung V30, V40, V50 and MLD were increased. In our study, lung V5 in VMAT was slightly increased (1A 51.37, 2A 51.03, and 7F 48.77), but V30 (1A 12.99, 2A 12.99, 7F 14.42) and MLD (1A 12.79, 2A 12.35 7F 12.52) were lower than in c-IMRT. The difference in V5, V30 and MLD may be due to that they avoided a certain angle in the VMAT plan with 2A, and this caused the reduction of the volume of irradiated lungs. One of the particular interesting phenomena in VMAT is the increase trends of mean or maximal radiation doses to the humeral head. This may be because that humeral head is adjacent to the target volume in the cervical and upper EC and the rotating mode of VMAT thus increased the irradiation volumes of the humeral head. However, clinical evidence on the acceptable humeral head constraints for IMRT remains scarce in literature. Nevertheless, according to the tolerated doses and clinical data of bone joints, such as femoral head and neck or temporomandibular joint[25,26], the maximum doses to humeral heads in the cervical EC (1A 34.00 Gy and 2A 33.82 Gy) or upper thoracic EC (1A 44.33 Gy and 2A 41.03 Gy) were considered acceptable, but we should pay attention to this performance and its potential risk.

Subsequently, the results obtained in cervical and upper thoracic EC were almost seen in middle and lower thoracic EC, with a PTV of smaller volume but surrounded by more lungs and heart. In thoracic and epigastric cases, except for a few complex cases, 5-7F of c-IMRT could meet most of the clinical dosimetric requirements. In our study, an ideal homogeneity was achieved in 5F or 7F of IMRT. With increase of the fields in c-IMRT or doubling arc in VMAT, dose distribution in PTV became more optimal in terms of better conformity and similar homogeneity. The trends were significantly different ($P < 0.05$) between VMAT(1A and 2A) and c-IMRT(5F and 7F). Recently, Van Benthuysen *et al*[27] demonstrated that VMAT had the advantage to decrease treatment times over c-IMRT, while providing similar OAR sparing and PTV coverage, but lower homogenous dose distribution in lower EC. In our study, we found that in the middle and lower thoracic EC, HI was similar in VMAT and c-IMRT. We did not find a significant difference between 5F or 7F IMRT and VMAT for these trends. For lesions in this region, more volumes of heart and lungs were involved in the irradiation area, mean doses to lungs and heart were elevated markedly and HI was also inferior to cervical and upper EC. Because lung tissue filled with air was significantly less dense than other body tissues, as a result of heterogeneity corrections in radiation treatment planning systems, optimization procedures produced substantial dose non-uniformity in PTV caused by the effect of surrounding lung tissues. To further optimize the dose in the target volume, dose heterogeneity was achieved by loosening the constraints on the maximum doses in PTV. It may result in insufficient dose in PTV or the creation of clinically significant hotspots in the PTV

and surrounding normal tissue structures. The National Comprehensive Cancer Network Guidelines recommend dose limits for selecting critical normal structures, i.e., the spinal cord doses should not exceed 45 Gy, and one-third of the heart should receive less than 50 Gy. The dosimetric parameters of lung injury risk were mainly studied on lung cancer irradiation, the increased risk of radiation pneumonitis correlated with heterogeneous parameters, such as MLD, the percentage of lung volume receiving at least 20 Gy (V20), 13 Gy (V13), 10 Gy (V10) or 5 Gy (V5), in which V20 was a recognized indicator confirmed by several studies. Based on pooled data from 540 patients irradiated for thoracic malignancy, the calculated risk of grade $\geqslant$ 2 pneumonitis was 43%, 18%, and 11% for the MLD of 24-36, 16-24 and 8-16 Gy, respectively. In our study, MLD was controlled below 16 Gy and it was acceptable. For conventionally fractionated regimens (2 Gy/fraction), V20 and MLD were the traditional parameters used to predict for lung toxicity, however, emerging data suggested that percentage of lung volume receiving lower doses may be predictive of pulmonary toxicity. VMAT plans offered the potential to significantly escalate the coverage of the low-dose area (V5 and V10) because all doses were deposited within the plane of the arc, instead of being spread out in non-coplanar directions. Mean V5 in VMAT was beyond 80% and it might increase the potential pulmonary toxicity. Wei *et al*[28] found that V30 > 46% and < 46% was associated with rates of pericardial effusion of 73% and 13%, respectively. The ischemic segments usually occur in volumes irradiated to a dose of 45 Gy or more. In our study, V40 and V50 were achieved in both VMAT and c-IMRT, but V30 was higher due to lower constrained priority. In VMAT, 1A achieved better results than 2A for less irradiation volumes of heart and lungs. In comparison with 5F or 7F, VMAT reduced V20 and V30 of lungs, and V30, V40 and V50 of heart. Besides, Hawkins *et al*[29] evaluated the capability of VMAT to reduce heart and cord dose while maintaining lung V20 < 20% in lower gastroesophageal tumors. IMRT (4F) and VMAT plans showed that VMAT provided a significant reduction in heart V30 (31% *vs* 55%) with a better CI in a shorter time. But V30 (2A 57.57 *vs* 5F 78.6) in our study was higher because our prescribed dose (60 Gy) was higher than theirs (54 Gy). NTCP of heart and lungs were common indicators in radiobiological assessment to indicate the tendency in plan comparison. VMAT had a trend with a lower NTCP of lungs and heart, but statistical significance only existed for lungs between 5F and 2A in lower EC, as for heart, between c-IMRT and 1A in middle thoracic EC, and between c-IMRT and 2A in lower EC ($P < 0.05$). Therefore, regarding the correlation between dosimetric parameters and OAR toxicity, we did find a superior trend in VMAT to c-IMRT. Wang *et al*[30] also conducted a planning comparison for EC between VMAT (1A and 2A) and 7F IMRT, and found that VMAT plan, especially using double arcs, could improve OAR sparing (lung V20 and V30, heart V30 and V40) and lower MUs without compromised target qualities as compared with IMRT. This was consistent with our findings.

VMAT reduced the number of required MU[31], because it was performed simultaneously with rotation by a dynamic MLC adaptation to the target volume during the rotation. Using double arcs, the rotation in clockwise and counter clock-wise directions allows diminished 25 s off-time between the two arcs[32]. The number of MU required is higher due to the sliding window technique. c-IMRT plans in this study offered wider than 15 cm in the direction of the MLC motion necessitating splitting into two sequences and doubling the number of fields. By contrast, one of the drawbacks of c-IMRT was the potential risk of second cancer. Theoretically, the significant reduction of MU by VMAT decreases scattered dose and may reduce the risk of secondary malignancies. The impact of irradiation of healthy tissues at low doses remains unresolved with the use of VMAT.

In conclusion, VMAT treatment plan was slightly better than c-IMRT in terms of PTV coverage. It provided an equivalent or better lungs and heart dose sparing, significant reduction of NTCP and MU per fraction. For cervical and upper EC, PTV was T-shaped across neck and chest, VMAT achieved fairly uniform dose distribution, but the 2A provided the best CI in all plans, and VMAT significantly increased the doses of humeral head. For middle and lower EC, in which PTV involved more lungs, VMAT plans offered the most conformal dose distribution and the potential to significantly escalate the coverage of lungs at low doses.

COMMENTS

Background

Esophageal cancer (EC) is very common in China and other developing countries. Radiotherapy is a major non-invasive treatment method with a high efficacy rate for EC. Innovative technologies have been developed in radiation delivery such as intensity-modulated radiotherapy (IMRT), and volumetric modulated arc therapy (VMAT) is a relatively new form of IMRT. There are several new interesting techniques in IMRT, but few evaluations have been available in term of their efficiency and safety.

Research frontiers

There have been few studies to compare the two radiotherapy techniques, particularly in EC. VMAT had already been investigated for some cancers. In this study, the authors further compared the VMAT plans and the conventional sliding window IMRT plans in EC of different anatomic regions.

Innovations and breakthroughs

Previous dose comparison studies showed that VMAT was able to produce similar or improved dose distributions, while achieving a reduction in treatment time and monitor units (MU). However, results are different in EC about dose to organs at risk (OAR) due to variable limitation conditions. Normal tissue complication probability (NTCP) of OAR in the VMAT plan, a common indicator in radiobiological assessment, is still not clear in EC. In the present study, the authors showed that VMAT, especially 2 arcs, slightly improved the OAR dose sparing for some organs, such as lungs and heart, and reduced the NTCP and MU with a better planning target volume coverage.

Applications

This study provides a new insight into better understanding of the VMAT plan characteristics in EC of different anatomical parts, and lays the foundation for further clinical studies in VMAT.

Terminology

IMRT is a three-dimensional conformal radiotherapy developed based on inverse planning optimization to modulate intensity beams using multi-leaf

collimator (MLC), this technique offers improvement in target dosimetric coverage. There are different IMRT delivery techniques, including "step and shoot", sliding window modes and a rotational technique (VMAT). Conventional sliding window IMRT, in which the leaves are adjusted with fixed gantry, is a common form which is being used in clinical practice. VMAT, in which dose rates, gantry speed and dynamic MLC motion are all variable during gantry arc rotation, is a novel form of IMRT in recent years.

Peer review

IMRT is developing a lot in Radiation Oncology Departments for a few years. There are several very interesting technics but still few real evaluations in terms of efficiency and safety. Comparing two technics is very interesting even if it's "only" a dosimetric comparison. This kind of comparisons of recent technics are not very frequent, particularly in EC but are developing a lot this last few years. The text of this manuscript is very clear and well presented.

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BRIEF ARTICLE

Nur-related receptor 1 gene polymorphisms and alcohol dependence in Mexican Americans

Ya-Ming Wei, Yan-Lei Du, Yu-Qiang Nie, Yu-Yuan Li, Yu-Jui Yvonne Wan

Ya-Ming Wei, Department of Blood Transfusion, Guangzhou First People's Hospital, Guangzhou Medical University, Guangzhou 510180, Guangdong Province, China
Yan-Lei Du, Yu-Qiang Nie, Yu-Yuan Li, Yu-Jui Yvonne Wan, Department of Gastroenterology and Hepatology, Guangzhou First People's Hospital, Guangzhou Medical University, Guangzhou 510180, Guangdong Province, China
Yu-Jui Yvonne Wan, Department of Pharmacology, Toxicology and Therapeutics, The University of Kansas Medical Center, Kansas City, KS 66160, United States
Author contributions: Wei YM, Wan YJ designed the study and assembled the data; Wei YM, Du YL drafted the article; and Nie YQ and Li YY critically reviewed the article.
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Correspondence to: Ya-Ming Wei, PhD, Department of Blood Transfusion, Guangzhou First People's Hospital, Guangzhou Medical University, Guangzhou 510180, Guangdong Province, China. weiyaming@163.com
Telephone: +86-20-81045129 Fax: +86-20-81048643
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Abstract

AIM: To investigate the association of polymorphisms of nur-related receptor 1 (*Nurr1*) and development of alcohol dependence in Mexican Americans.

METHODS: Peripheral blood samples were collected from 374 alcoholic and 346 nonalcoholic Mexican Americans; these two groups were sex- and age-matched. Sample DNA was extracted and genomic DNA was amplified by polymerase chain reaction. The *-2922(C) 2-3* polymerase chain reaction products were digested with *Sau96I*, alleles of *1345(G/C)*, and *-1198(C/G)* in the regulatory region as well as *Ex+132 (G/T/A/C)* and *Ex+715(T/-)* in exon 3 were studied by sequencing.

RESULTS: The *C2/C2, C2/C3, C3/C3* genotype distribution of *-2922(C) 2-3* was 34.4%, 38.2% and 27.5% in the nonalcoholic group compared to 23.3%, 51.2% and 25.4% in the alcoholic group (P = 0.001). The *C/C, C/G, G/G* genotype distribution of *-1198(C/G)* was 23.5%, 46.1% and 30.3% in the nonalcoholic group compared to 13.9%, 50.9% and 35.3% in the alcoholic group (P = 0.007). However, the *-1345 (G/C)*, *Ex3+132(G/T/A/C)* and *Ex3+715(T/-)* alleles were not polymorphic in Mexican Americans, and all those studied had *G/G, G/G* and *T/T* genotype for these three alleles, respectively. The *-2922(C) 2-3* did not show allele level difference between alcoholic and nonalcoholic individuals, but *-1198 (C/G)* showed a significant allele frequency difference between alcoholic (39.3%) and nonalcoholic (46.6%) populations (P = 0.005). Excluding obese individuals, significant differences were found at both genotypic and allelic levels for the *-2922(C) 2-3* polymorphism (P = 0.000 and P = 0.049) and the *-1198 (C/G)* polymorphism (P = 0.008 and P = 0.032) between nonobese alcoholics and nonobese controls. Excluding smokers, a significant difference was found only at the genotypic level for the *-2922(C) 2-3* polymorphism (P = 0.037) between nonsmoking alcoholics and nonsmoking controls, but only at the allelic level for the *-1198(C/G)* polymorphism (P = 0.034).

CONCLUSION: Polymorphisms in the regulatory region of *Nurr1* are implicated in pathogenesis of alcohol dependence and the *Nurr1*/dopamine signaling pathway might be important for this dependence development in Mexican Americans.

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Key words: Nur-related receptor 1; Polymorphism; Alcohol dependence; Obesity; Smoking; Nuclear receptor

Peer reviewer: Sung-Gil Chi, Professor, School of Life Sciences and Biotechnology, Korea University, No. 301, Nok-Ji Building, Seoul 136-701, South Korea

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INTRODUCTION

It is widely accepted that genetic factors play an important role in the development of alcohol dependence. Family clustering surveys have shown that alcohol dependence rates are higher among persons who are biologically related to an alcoholic[1], and that a family alcohol history is a strong predictor of alcohol dependence[2]. Twin pair studies have revealed a significantly greater concordance rate for alcohol dependence in monozygotic compared with dizygotic twins[3]. Half sibling and adoption studies have demonstrated that half brothers with different fathers and adopted sons of alcoholic men show a rate of alcohol dependence more like that of the biological father than that of the foster father[4]. On the other hand, there is no classic Mendelian pattern of inheritance for alcohol dependence. Environmental factors are also linked to alcohol dependence. Thus, alcohol dependence is a polygenic complex disorder resulting from an intricate interaction of multiple genes and various environmental factors.

Genes encoding alcohol metabolizing enzymes alcohol dehydrogenase (*ADH1B)* and aldehyde dehydrogenase (*ALDH2*) are the only genes that have been firmly linked to alcoholism[5,6]. However, data suggest that genes involved in the brain reward pathways are also strong candidates for a predisposition to alcohol dependence. These pathways, in particular, those utilizing dopamine and opioids as neurotransmitters, mediate positive reinforcement of activities, such as eating, love and reproduction. When engaging in such activities, the "natural reward" center releases dopamine from brain pathways in the nucleus accumbency and frontal cortex[7,8]. However, these same pathways can also be activated by "unnatural rewards" such as alcohol abuse. Only a minority of individuals become addicted to these various substances and certain types of behavior, therefore, it should be possible to identify factors, such as genes, that distinguish them from others not affected. Dopamine is the primary neurotransmitter in the reward pathway, genes that control dopamine signaling, including dopamine receptors and transporters, are particularly tempting candidates. Alcohol dependence is likely to be a polygenic and multifactorial disease, therefore, certain genes might have a small effect, whereas others have a greater impact in terms of increasing the risk for this disorder.

Our previous studies have shown that certain alleles of the brain dopamine receptors 2 and 4 (*DRD2* and *DRD4*) and of the serotonin transporter (*5-HTT*) as well as of opioid receptor are associated with alcohol dependence[9-13]. These findings indicate the importance of polymorphism of the gene in the reward pathway in contributing to the development of alcohol dependence in Mexican Americans.

Alcohol consumption, compulsive overeating and smoking are all associated with dysfunction of rewards pathways; there might be common risk factors in reward genes for these behaviors. *DRD2 TaqI A1* allele was identified at a higher frequency in alcoholic and nonalcoholic smokers than nonsmoking controls[14]. Smoking and obesity related to overeating might serve as confounding factors when association is analyzed between reward genes and alcohol dependence.

The Nur-related receptor 1 (*Nurr1*), *NR4A2*, is a transcription factor in the orphan nuclear receptor family[15-17], and is important for development of dopaminergic neurons. Ablation of *Nurr1* leads to agenesis of midbrain dopaminergic neurons as demonstrated by an absence of dopaminergic cell markers including tyrosine hydroxylase, as well as a loss of striatal dopamine neurotransmitter[18]. *Nurr1* knockout mice failed to develop ventral mesencephalic dopaminergic neurons and died within 12-48 h[19]. In addition, *Nurr1* increases expression of the human dopamine transporter gene in the mature brain; whereas other members of the nerve growth factor-induced clone B subfamily of nuclear receptors have lesser or even no effects[19]. Expression of *Nurr1* continues in mature dopaminergic neurons during adulthood, suggesting that *Nurr1* is also required for normal function of mature dopaminergic neurons[20]. Thus, *Nurr1* is an upstream signaling molecule for regulating the dopamine pathway and plays a broad-spectrum role in brain development.

Nurr1 involvement in the regulation of the dopaminergic system makes it a good candidate to study neuropsychiatric disorders. Several polymorphisms in the gene have been identified: *-469delG*, *M97V*, *H103R*, *DY122*, *-2922(C)2-3*, *IVS6+17* approximately *+18insG* and *EX8+657(CA)9-10*[21-24]. Among these polymorphisms, *-2922(C)2-3*, *IVS6+17* approximately *+18insG* and *EX8+657* variants are common (frequency of minor alleles > 15%) in both Caucasian and Asian populations. Two variants (*-291Tdel* and *-245T→G*) of *Nurr1* are associated with Parkinson's disease[25]. However, another study failed to identify any of the described variants in Parkinson's patients or controls[26]. In the regulatory region of the *Nurr1* gene, at least five polymorphic sites [*P1(A/T*, reference SNP number rs1462374), *P2(G/C)*, *P3(C/G)*, *P4(C/A/G)*, *P5(del C)*] have been identified, with no significant association between the genotype or allele frequency of these variants and schizophrenia or Parkinson's disease. In addition, *P2(G/C)*, *P3(C/G)*, and *P5(del C)* are in linkage disequilibrium (LD) with each other[27]. Further investigation of common *Nurr1* variants [*-2922(C)2-3*, *IVS6+17* approximately *+18insG*, *EX8+657(CA) 9-10*] has not supported their pathogenic role for schizophrenia among Japanese individuals[28]. There are two coding synonymous polymorphism sites in exon 3 (rs16840266) and exon 5 (rs61748236), and three coding nonsynonymous polymorphism sites in exon 3 (rs36083712 and rs35100271) and exon 8 (rs61729997); some other rare missense mutations have been studied in psychiatric disorders[22], but none of them has been found to be associated with alcohol addiction.

The role of *Nurr1* in alcohol dependence was studied in Japanese individuals[29], and polymorphisms including *-2922(C) 2-3* and *EX8 +657(CA)9-10* were examined. They have reported that the genotypic distribution of these two polymorphisms was in Hardy-Weinberg equilibrium in the controls and alcoholics. The allele frequency of the *(C) 2* and *(CA) 9* and allele in the alcoholics was similar to that in the controls. Significant LD between these two polymorphisms was observed in both controls ($P = 0.007$) and alcoholics ($P < 0.0001$), but the LD was much stronger in alcoholics than in controls (D' = 0.84 *vs* D' = 0.30). There was a significant difference in haplotype distributions between the alcohol dependence and control groups. The haplotypic association was not based on an increased frequency of a specific haplotype, but rather based on stronger LD in alcoholics. This finding implies that more recent ancestral chromosome sharing by the alcoholics than by the controls, and that the *Nurr1* locus is one of the genome regions that contribute to alcohol dependence.

Nurr1 is involved in the regulation of the dopaminergic system, which is the primary neurotransmitter in the reward pathway. The dopamine signaling can be activated by alcohol. Thus, we hypothesize the presence of an association between the polymorphism of the *Nurr1* gene and alcohol dependence. We selected three alleles in the regulatory region of the *Nurr1* gene that include *-2922(C)2-3*, *-1345(G/C)* and *-1198(C/G)* (promoter P2 and P3 in reference literature from Andrea Carmine) as well as coding synonymous *Ex+132(G/T/A/C*, rs16840266) and coding nonsynonymous *Ex+715(T/-*, rs35100271) in exon 3. These alleles are supposed to contribute to the function of *Nurr1* and may have an impact on alcohol dependence in Mexican Americans.

MATERIALS AND METHODS

Participants

All participants in this project were enrolled from unrelated Mexican Americans who lived in Los Angeles County, CA. The study cohorts included 374 alcoholics and 346 nonalcoholics, and the ratio of cases to controls was about 1.08. The groups were sex- and age-matched. All subjects provided informed consent for their participation in this study. This study was approved by the University of Kansas Medical Center Human Subjects Committee. All participants were investigated, diagnosed, and assigned based on the Diagnostic and Statistical Manual of Mental Disorder, Fourth Edition (DSM-IV). The alcoholic participants were interviewed by the Semi-Structured Assessment for the Genetics of Alcohol dependence II in English or Spanish[30]. The inclusion criteria for alcoholic participants included: (1) the ability to give informed consent; (2) between 21 and 76 years of age; (3) no less than three of four biological grandparents of Mexican heritage; (4) fluency in either Spanish or English; (5) no current use of other substances (except tobacco and caffeine), or history of such use within the past 6 mo; (6) no current or past diagnosis of mental illness such as schizophrenia, schizophrenia disorder, schizoaffective disorder, schizotypal disorder, major depression, bipolar disorder, or Parkinson's disease; and (7) no other clinical unacceptable disease based on physical examinations. The inclusion criteria for nonalcoholic participants included: (1) no current or history of diagnosis of DSM-IV alcohol dependence or alcohol abuse; and (2) no other clinical unacceptable disease based on physical examinations. The interview information also covered body weight, height, smoking status, tea and coffee intake, as well as marriage, and education status. Body mass index (BMI) equals body weight in kilograms divided by height in square meters (kg/m^2). Smoking status was defined as having smoked one or more cigarettes per day during the past 30 d[31].

Genotyping

Participants' peripheral blood samples were collected into tubes containing K_2-EDTA. Genomic DNA was extracted with GeneCatcher gDNA Blood kits (Invitrogen, Carlsbad, CA, United States). The primers used for PCR were: *-2922(C)2-3*, forward: 5'-AAAAGGGGATGAACCGGGTAGG-3', reverse: 5'-TTTTCCGAAAGAGGTGTGACCT-3'; *-1345(G/C)* and *-1198(C/G)*, forward: 5'-ATCCCGAATAGTTCCACGGAG-3', reverse: 5'-CACGAGTTTTAAGGGAATAAATCG-3'; *Ex3+132(G/T/A/C)* and *Ex3+715(T/-)*, forward: 5'-GCTGAGTGTGTTATCACCTGTTT-3', reverse: 5'-GCTGAGTGTGTTATCACCTGTTT-3'.

PCR amplification was carried out in a 25-μL reaction mix containing 100 ng genomic DNA, 1× Go Taq Flexi buffer, 0.2 μmol/L each primer, 0.2 mmol/L dNTP, 2.0 mmol/L $MgCl_2$, 0.2 mmol/L dGTP, and 1 U GoTaq DNA polymerase. The thermal cycling conditions were set at 95 ℃ for 5 min, then 35 cycles for 30 s at 95 ℃, 30 s at 50 ℃ (for the three alleles in the regulatory region) or 55 ℃ (for the two alleles in exon 3), and 60 s at 72 ℃, with a final extension step of 10 min at 72 ℃. PCR products were examined on a 2% agarose gel.

The *-2922(C)2-3* PCR products were digested with *Sau96I*, which generated 176- and 158-bp fragments for wild-type alleles. Positive and negative digestion controls were set in each PCR and each agarose gel to ensure correct digest results. Retest was needed when results could not be read clearly. Other genotyping was studied by sequencing. All samples were sequenced in two directions. Results where two direction sequences did not match each other were excluded. Sequencing results were read by Finch TV (1.4 Version, Geospiza, Inc.) graphical viewer.

Statistical analysis

Statistical analyses were performed with SPSS Version 15.0 software (SPSS Inc., Chicago, IL, United States). Two-sided analyses were conducted and $P < 0.05$ was used as the significance threshold. Pearson's χ^2 tests were used to compare sex, BMI, smoking, genotype, and allele distribution between alcoholics and their controls.

Table 1 Genotype and allele frequency of the *-2922(C)2-3* and *-1198(C/G)* allele in Mexican American alcoholics and nonalcoholics *n* (%)

Groups	*-2922(C)2-3* allele					*-1198(C/G)* allele				
	Genotype			Allele frequency	Total	Genotype			Allele frequency	Total
	(C)2/(C)2	*(C)2/(C)3*	*(C)3/(C)3*	*(C)3*		*C/C*	*C/G*	*G/G*	*C*	
Non-alcoholic	119 (34.4)	132 (38.2)	95 (27.5)	322 (46.5)	346	76 (23.5)	146 (46.1)	98 (30.3)	298 (46.6)	320
Alcoholic	87 (23.3)	192 (51.3)	95 (25.4)[a]	382 (51.1)	374	48 (13.9)	176 (50.9)	122 (35.3)[a]	272 (39.3)[b]	346

[a]$P = 0.007$; [b]$P = 0.005$ *vs* nonalcoholic.

RESULTS

Population characteristics

There were 374 alcoholics and 346 nonalcoholic controls. The alcoholic group had 299 (79.9%) men and 75 (20.1%) women, and the nonalcoholic control group had 260 (75.1%) men and 86 (24.9%) women. The number of young (≤ 30 years), middle-aged (30-60 years), and old (> 60 years) participants in the control group was 93 (26.9%), 245 (70.8%), and 8 (2.3%), respectively, and 99 (26.5%), 266 (71.1%), and 9 (2.4%) in the alcoholic group. The mean ± SD age of the alcoholics and controls was 38.3 ± 10.5 years (range: 21-76 years) and 37.3 ± 10.4 years (range: 19-65 years), respectively. No significant differences were found between the two cohorts regarding sex or age distribution with Pearson's χ^2 test.

BMI is the measurement of choice to determine obesity, and the clinical diagnosis of obesity is BMI ≥ 30 kg/m^2. The BMI was not significantly different between the alcoholic and nonalcoholic groups [BMI < 30 kg/m^2, 198 (57.6%) *vs* 213 (63.6%); BMI ≥ 30 kg/m^2, 146 (42.4%) *vs* 122 (36.4%), $P = 0.108$]. However, the smoker distribution was significantly different between the alcoholic and nonalcoholic groups [non-smoker, 197 (52.7%) *vs* 282 (82.7%); smoker, 177 (47.3%) *vs* 59 (17.3%), $P = 0.000$]. The alcoholic group had more smokers than the control group had.

Genotypes of -2922(C)2-3, -1345(G/C), -1198(C/G), Ex3+132(G/T/A/C) and Ex3+715(T/-) in Mexican Americans

The genotypes of *-2922(C)2-3*, *-1345(G/C)*, *-1198(C/G)*, *Ex3+132(G/T/A/C)* and *Ex3+715(T/-)* were studied in Mexican American alcoholics and controls. The *-2922(C)2-3* and *-1198(C/G)* alleles were polymorphic in Mexican Americans, but *-1345(G/C)*, *Ex3+132(G/T/A/C)* and *Ex3+715(T/-)* were shown to be present in the studied population (*G/G*, *G/G*, and *T/T*, respectively). These results are consistent with reports which come from Swedish[27], Japanese[29] and Canadian[32] populations with neurological disorders. This is believed to be the first research reporting the absence of polymorphisms in Mexican Americans at the three genomic sites mentioned above.

The genotypes of *-2922(C)2-3* and *-1198(C/G)* as well as their minor allele frequencies are shown in Table 1. The Hardy-Weinberg equilibrium *P* values of *-2922(C)2-3* and *-1198(C/G)* in alcoholics were 0.6914 and 0.2751, respectively, and the *P* values for these two alleles in nonalcoholic controls were 0.00002 and 0.1854. The genotype distribution of the *-2922(C)2-3* allele was significantly different between alcoholics and nonalcoholics ($P = 0.001$); however, there was no significant difference at the allelic level. The genotype as well as the allele frequency of the *-1198(C/G)* allele was significantly different between alcoholics and nonalcoholics ($P = 0.005$ and $P = 0.007$), suggesting the importance of sequence variations in the regulatory region of the *Nurr1* gene in differentiating these two cohorts. In addition, the *-2922(C)2-3* and *-1198(C/G)* alleles showed a strong LD (D' = 0.88) in the alcoholic population, but not in the nonalcoholic population.

Association of -2922(C)2-3 and -1198(C/G) with alcohol dependence after controlling for confounding effect of smoking and obesity

Alcoholics and nonalcoholic controls were stratified according to the smoking and obesity status. When obese individuals were excluded, a significant difference was found at both the genotypic and allelic level between non-obese alcoholics and non-obese controls, for the *-2922(C)2-3* polymorphism (Table 2, $P = 0.000$ and $P = 0.049$) and *-1198(C/G)* polymorphism (Table 3, $P = 0.008$ and $P = 0.032$). When smokers were excluded, between alcoholics and controls, a significant difference was found only at the genotypic level for the *-2922(C)2-3* polymorphism (Table 2, $P = 0.037$), but only at the allelic level for the *-1198(C/G)* polymorphism (Table 3, $P = 0.034$).

DISCUSSION

Hispanics are one of the fastest growing ethnic groups in the United States and were expected to become the largest minority group by the year 2010[33]. About two-thirds of Hispanic population is of Mexican American or Mexican origin. These population demographics have shown evidence of a serious problem of alcohol dependence with a prevalence rate of heavy drinking in Mexican American men that is three times higher than in non-Hispanic male populations[34]. Excessive alcohol drinking carries a high risk of developing various types of chronic diseases. Alcohol-related problems in this population, such as alcoholic liver disease, malignant neoplasms, psychiatric conditions, neurological impairment, and cardiovascular disease show a significantly higher

Table 2 Genotype and allele frequency of the *-2922(C)2-3* allele in Mexican American alcoholics and non-alcoholics controlling for confounding effect of smokers and obesity *n* (%)

Groups	Genotype			Allele frequency
	(C)2/(C)2	*(C)2/(C)3*	*(C)3/(C)3*	*(C)3*
Non-alcoholic				
BMI < 30 kg/m^2 (213)	73 (34.3)	78 (36.6)	62 (29.1)	202 (47.4)
BMI ≥ 30 kg/m^2 (122)	41 (33.6)	48 (39.3)	33 (27.0)	114 (46.7)
Alcoholic				
BMI < 30 kg/m^2 (198)	37 (18.7)	107 (54.0)	54 (27.3)[a]	215 (54.3)[b]
BMI ≥ 30 kg/m^2 (146)	37 (21.5)	74 (50.7)	35 (25.9)	144 (49.3)
Non-alcoholic				
Non-smokers (282)	100 (35.5)	101 (35.8)	81 (28.7)	263 (46.6)
Smokers (59)	17 (28.8)	29 (49.2)	13 (22.0)	55 (46.6)
Alcoholic				
Non-smokers (197)	49 (24.9)	88 (44.7)	60 (30.5)[c]	208 (52.8)
Smokers (177)	38 (21.5)	104 (58.8)	35 (19.8)[d]	174 (49.2)

[a]*P* = 0.000 *vs* nonalcoholic body mass index (BMI) < 30 kg/m^2; [b]*P* = 0.049 *vs* nonalcoholic BMI < 30 kg/m^2; [c]*P* = 0.037 *vs* non-alcoholic non-smokers; [d]*P* = 0.016 *vs* alcoholic non-smokers.

Table 3 Genotype and allele frequency of the *-1198(C/G)* allele in Mexican American alcoholics and non-alcoholics controlling for confounding effect of smoking and obesity *n* (%)

Groups	Genotype			Allele frequency
	C/C	*C/G*	*G/G*	*C*
Non-alcoholic				
BMI < 30 kg/m^2 (199)	54 (27.1)	81 (40.7)	64 (32.2)	189 (47.5)
BMI ≥ 30 kg/m^2 (115)	21 (18.3)	62 (53.9)	32 (27.8)	104 (45.2)
Alcoholic				
BMI < 30 kg/m^2 (181)	26 (14.4)	92 (50.8)	63 (34.8)[a]	144 (39.8)[b]
BMI ≥ 30 kg/m^2 (136)	18 (13.2)	73 (53.7)	45 (33.1)	109 (40.1)
Non-alcoholic				
Non-smokers (265)	61 (23.0)	122 (46.0)	82 (30.9)	244 (46.0)
Smokers (55)	15 (18.3)	24 (53.9)	16 (27.8)	54 (49.1)
Alcoholic				
Non-smokers (185)	27 (14.6)	90 (48.6)	68 (36.8)	144 (38.9)[c]
Smokers (161)	21 (13.0)	86 (53.4)	54 (33.5)[d]	128 (39.7)

[a]*P* = 0.008 *vs* nonalcoholic body mass index (BMI) < 30 kg/m^2; [b]*P* = 0.032 *vs* nonalcoholic BMI < 30 kg/m^2; [c]*P* = 0.034 *vs* nonalcoholic non-smokers; [d]*P* = 0.05 *vs* non-alcoholic smokers.

incidence than when compared with those from other ethnic backgrounds[35]. Apart from environmental factors, using family, twin pair, half sibling, and adoption studies, alcohol dependence has been proved to be a polygenic disorder[1-4].

Candidate gene associations approach has been widely used to explore related genetic factors. We have previously studied genes involved in both alcohol metabolism and reward pathways in Mexican American alcoholics and have established associations between certain phenotypes of alcohol dependence and the polymorphism of these genes[9-13,36,37]. We showed that most Mexican Americans carry the *ADH1B*1* (95%) and *ALDH2*1* (99.4%) genes. Thus, the *ADH1* and *ALDH2* genotypes do not distinguish those more prone to alcohol drinking from those who are not[9]. Our data also showed the importance of polymorphism of *DRD2 (-141C Del/Ins)* and *5-HTTLPR* in contribution to alcohol dependence[9-12]. Other components of the dopamine pathway have also been associated with alcohol dependence. *DRD4 VNTR* genotypes without the 7-repeat allele have been found to be risk factors for alcohol dependence in Mexican Americans[13]. In the current study, to the best of our knowledge, we reported for the first time a significant association between the polymorphisms in the promoter region of the *Nurr1* gene and alcohol dependence in Mexican Americans. Our findings indicated that polymorphisms of *-2922(C)2-3* and *-1198(C/G)* are associated with alcohol dependence in the Mexican American population, even when the confounding effects of smoking and obesity were controlled, suggesting the reliability of our findings. The frequency of the *-1198 G* allele was significantly higher in alcoholics than in controls, suggesting that the *G* allele of *-1198(C/G)* might potentially have a pathogenic effect on alcohol drinking. The functional significance of these two polymorphisms still needs to be explored. Taken together, since *Nurr1* is upstream of the dopamine pathway, our data indicate the importance of *Nurr1*/dopamine signaling in alcohol abuse in this important minority group.

Smoking and obesity might represent significant confounding factors in the relationship between risk factors and alcohol dependence. Alcohol and constituents of tobacco are potent inducers of *CYP2E1*. When smokers were excluded from both the control and alcoholic groups, the **5B* allele of the *CYP2E1* gene was significantly associated with alcohol dependence[38]. Similar findings were observed for the *DRD2 -141C Ins/Del* allele[10]. In the present studied population, 47.3% of alcoholics and 17.3% of nonalcoholics were smokers. The distribution of smokers and nonsmokers in alcoholic and nonalcoholic groups showed a significant difference. When all participants were considered, distribution of both *-2922(C)2-3* and *-1198(C/G)* polymorphisms showed significant differences between the alcoholic and nonalcoholic groups. When smokers were excluded, the association of alcohol dependence with these two polymorphisms remained. When obesity was considered, *-2922(C)2-3* and *-1198(C/G)* polymorphisms were associated with alcohol dependence in the non-obese groups. Associations of these two polymorphisms and alcohol dependence are consistently found in the nonsmoking and non-obese group, therefore, this increases the reliability of our findings.

In conclusion, the polymorphism of *-2922(C)2-3* and *-1198(C/G)* in the regulatory region of the *Nurr1* gene were shown to be associated with alcohol dependence in Mexican Americans. The functional significance of these two polymorphisms still needs to be studied. Alcohol dependence is a multifactorial disease, therefore, it is also important to study the interaction of *Nurr1* with other components of the dopamine signaling pathway in contributing to alcohol dependence, which might lead to effective treatment strategies.

COMMENTS

Background

It is widely accepted that genetic factors play an important role in the development of alcohol dependence. Family clustering surveys have shown that alcohol dependence rates are higher among persons who are biologically related to an alcoholic. Nur-related receptor 1 (*Nurr1*) is a transcription factor in the orphan nuclear receptor family that can regulate dopamine neurotransmission and influence the expression of genes important for human brain development.

Research frontiers

Nurr1 regulates the dopaminergic system. Several polymorphisms in the gene have been identified. Two variants (*-291Tdel* and *-245T→G*) of *Nurr1* are associated with Parkinson's disease. The *Nurr1* locus is located in the genome region that contributes to alcohol dependence. Thus, the authors hypothesize the presence of an association between polymorphism of the *Nurr1* gene and alcohol dependence.

Innovations and breakthroughs

Several polymorphisms of *Nurr1* have been identified: *-469delG*, *M97V*, *H103R*, *DY122*, *-2922(C)2-3*, *IVS6+17* approximately *+18insG* and *EX8+657(CA)9-10*. *-2922(C)2-3*, *IVS6+17* approximately *+18insG* and *EX8+657* variants are common in both Caucasian and Asian populations. Two variants (*-291Tdel* and *-245T→G*) of *Nurr1* are associated with Parkinson's disease. Further investigation of common *Nurr1* variants did not support their pathogenic role, and none was found to be associated with alcohol addiction. In the present study, the authors showed that genotype distribution of *-2922(C) 2-3* and *-1198(C/G)* was significantly different between nonalcoholic and alcoholic Mexican Americans. The *-1198C* frequency was found to be significantly higher in nonalcoholics than that in alcoholics. However, the *-1345(G/C)*, *Ex3+132(G/T/A/C)*, and *Ex3+715(T/-)* alleles were not polymorphic in Mexican Americans; all the studied Mexican Americans had *G/G*, *G/G and T/T* genotype for these three alleles, respectively.

Applications

This study suggested that polymorphisms in the regulatory region of the *Nurr1* gene are implicated in pathogenesis of alcohol dependence of Mexican Americans. The *Nurr1*/dopamine signaling pathway might be important for the development of alcohol dependence in Mexican Americans.

Terminology

NR4A2 is a transcription factor in the orphan nuclear receptor family, and is important for development of dopaminergic neurons. *Nurr1* is an upstream signaling molecule for regulating the dopamine pathway and plays a broad-spectrum role in brain development.

Peer review

This is a good descriptive study in which the authors analyzed the polymorphisms of the *Nurr1* gene in 374 alcoholic and 346 nonalcoholic Mexican Americans. They found that the genotype distributions of *-2922(C) 2-3* and *-1198(C/G)* were different between alcoholics and non-alcoholics. The presented data suggest that the *Nurr1*/dopamine signaling pathway might be important for the development of alcohol dependence in Mexican Americans.

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BRIEF ARTICLE

Antifibrotic effect of N-acetyl-seryl-aspartyl-lysyl-proline on bile duct ligation induced liver fibrosis in rats

Lei Zhang, Lei-Ming Xu, Yuan-Wen Chen, Qian-Wen Ni, Min Zhou, Chun-Ying Qu, Yi Zhang

Lei Zhang, Lei-Ming Xu, Yuan-Wen Chen, Qian-Wen Ni, Min Zhou, Chun-Ying Qu, Yi Zhang, Digestive Endoscopic Diagnosis and Treatment Center, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200092, China

Author contributions: Zhang L, Xu LM, Chen YW designed research; Zhou M and Qu CY contributed new reagents/analytic tools; Zhang Y and Ni QW analyzed data; and Zhang L, Zhou M and Xu LM wrote the paper.

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Correspondence to: Dr. Lei-Ming Xu, Chief Physician, Digestive Endoscopic Diagnosis and Treatment Center, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200092, China. leiming.xu@yahoo.com.cn
Telephone: +86-21-65790000 Fax: +86-21-65790000

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Abstract

AIM: To investigate the preventive effect of N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) on bile duct ligation (BDL)-induced liver fibrosis in rats.

METHODS: Liver fibrosis in rats was induced by BDL and AcSDKP was infused subcutaneously for 2 wk *via* a osmotic minipump (Alzet 2ML4) immediately after BDL operation. After scarifying, serum and liver specimens were collected. Hematoxylin and eosin staining, Sirius red staining, enzyme linked immunosorbent assay, Western blot or real-time polymerase chain reaction were used to determinate liver functions, histological alterations, collagen deposition, mRNA expression of markers for fibroblasts, transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-7 (BMP-7).

RESULTS: When compared to model rats, chronic exogenous AcSDKP infusion suppressed profibrogenic TGF-β1 signaling, α-smooth muscle actin positivity (α-SMA), fibroblast specific protein-1 (FSP-1) staining and collagen gene expression. Col Ⅰ, Col Ⅲ, matrix metalloproteinase-2, tissue inhibitors of metalloproteinase-1 and tissue inhibitors of metalloproteinase-2 mRNA expressions were all significantly downregulated by AcSDKP infusion (2.02 ± 1.10 *vs* 14.16 ± 6.50, 2.02 ± 0.45 *vs* 10.00 ± 3.35, 2.91 ± 0.30 *vs* 7.83 ± 1.10, 4.64 ± 1.25 *vs* 18.52 ± 7.61, 0.46 ± 0.16 *vs* 0.34 ± 0.12, respectively, $P < 0.05$). Chronic exogenous AcSDKP infusion attenuated BDL-induced liver injury, inflammation and fibrosis. BDL caused a remarkable increase in alanine transaminase, aspartate transaminase, total bilirubin, and prothrombin time, all of which were reduced by AcSDKP infusion. Mast cells, collagen accumulation, α-SMA, TGF-β1, FSP-1 and BMP-7 increased. The histological appearance of liver specimens was also improved.

CONCLUSION: Infusion of exogenous AcSDKP attenuated BDL-induced fibrosis in the rat liver. Preservation of AcSDKP may be a useful therapeutic approach in the management of liver fibrosis.

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Key words: N-acetyl-seryl-aspartyl-lysyl-proline; Liver fibrosis; Transformating growth factor-β1; α-smooth mucle actin; Bone morphological protein-7; Fibroblast specific protein-1; Epithelial-mesenchymal transition

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INTRODUCTION

N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is an endogenous tetrapeptide normally present in the plasma and organs of humans and experimental animals[1-3]. It is released locally in tissues from its precursor thymosin-β4 (Tβ4) most likely by prolyl oligopeptidase (POP), a serine proteinase found in mammalian tissues[4,5]. AcSDKP is cleaved to an inactive form by the NH_2-terminal catalytic domain of angiotensin converting enzyme (ACE)[6].

Originally described as a natural inbibitor of hematopoietic stem cell proliferation, AcSDKP is now recognized as a critical negative regulator for extracellular matrix (ECM) accumulation in organs under both physiological and pathological conditions. Decreased basal levels of endogenous AcSDKP by ACE over expression or by POP inhibitors promote cardiac fibrosis and/or glomerulosclerosis[7,8]. Exogenous AcSDKP infusion reduces collagen deposition in rats heart and/or kidney under hypertensive and ischemic conditions[9]. AcSDKP also mediates the antifibrogenic effect of ACE inhibitors in the heart[10]. The mechanism of action of AcSDKP includes suppression of inflammation, ECM-producing cell proliferation, collagen production, and more importantly transforming growth factor-β1 (TGF-β1) signaling[7-9,11,12].

Indeed, these key cellular and molecular mechanisms are critical in regulating ECM accumulation in multiple organs, in particular the liver[13]. Moreover, ACE inhibition is beneficial in several liver fibrosis models where there is increased ACE activity and potentially excessive AcSDKP degradation, Tβ4 and significant POP activity are present in the liver, where AcSDKP is produced locally, and may play a role in the regulation of hepatic cell responses *in vivo*[14,15].

Our previous studies had revealed that AcSDKP ameliorated carbon tetrachloride (CCl_4)-induced liver fibrosis and liver functions in the rat liver. The current study was aimed to investigate the preventive effect of AcSDKP on bile duct ligation (BDL)-induced liver fibrosis in rats. The potential mechanisms involved were also examined.

We explored the effects of AcSDKP on liver fibrosis by infusion of exogenous AcSDKP into the BDL rat models. Our results demonstrate that exogenous AcSDKP preserves basal levels of AcSDKP in the liver and significantly reduces the development of liver fibrosis in this model. Based on these findings, we propose that AcSDKP plays an important role in attenuating liver fibrosis. The underlying mechanisms may involve decreased production of profibrotic cytokines and reduced collagen expression and accumulation.

MATERIALS AND METHODS

Materials

BDL-induced rat liver fibrosis models: all animal handling and experimental procedures were approved by the Animal Care and Use Committee of the Shanghai Jiaotong Uinversity School of Medicine. Male Sprague-Dawley rats (200-250 g) were obtained from the Shanghai Experimental Animal Center (Shanghai, China). The rat model of liver fibrosis was induced by BDL. Upon sacrifice, blood was collected and serum and/or plasma were obtained. Liver tissue was either fixed in 10% neutral buffered formalin, frozen in optimal cutting temperature, or snap frozen in liquid nitrogen and stored at -80 ℃.

Experiment: AcSDKP-infused BDL-treated rats and the BDL model were established as above (n = 8-10). AcSDKP-infused BDL-treated rats were infused with AcSDKP at 800 μg/kg per day through a subcutaneous osmotic minipump (Alza Corp, Palo Alto, CA) beginning simultaneously with BDL. Rats were sacrificed at 2 wk. This dosage was used because it increased plasma AcSDKP to a concentration similar to that induced by captopril (100 μg/kg per day, 3- to 5-fold-change), without any adverse effect on the circulatory system[9].

Serum assays

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and albumin in serum and prothrombin time in plasma were measured using an automated analyzer.

Histological analysis

Formalin-fixed paraffin sections of the liver were stained with hematoxylin and esosin for pathological analysis or Sirius red for collagen. Collagen was quantified with Image Quant 5.1 software as previously described[16]. Positive cells were enumerated in 10 randomly selected fields at 400 × magnification.

Gene expression

Total RNA was extracted from livers using Trizol and was reverse-transcribed using an iscript cDNA synthesis kit. Real-time polymerase chain reaction (PCR) was performed on an iCycler system using the SYBR green Master Mix. Primer specificity was confirmed by sequencing PCR products. β-actin was the internal control. Data were presented according to the $\Delta\Delta C_t$ method.

Western blot

Frozen liver tissue was homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitors. A full list of antibodies is available in Supplemental data. Western blot was performed as previously described[17]. Bands were quantified by Scion Image 4.0.3. The loading control was tubulin.

Hydroxyproline content

Hydroxyproline content in liver tissue was determined as previously described[18].

Statistical analysis

Data are expressed as means ± SE. Comparisons were performed using analysis of variance. Least significant difference procedure analyses were performed when > 2 groups were present. $P < 0.05$ was considered statistically significant.

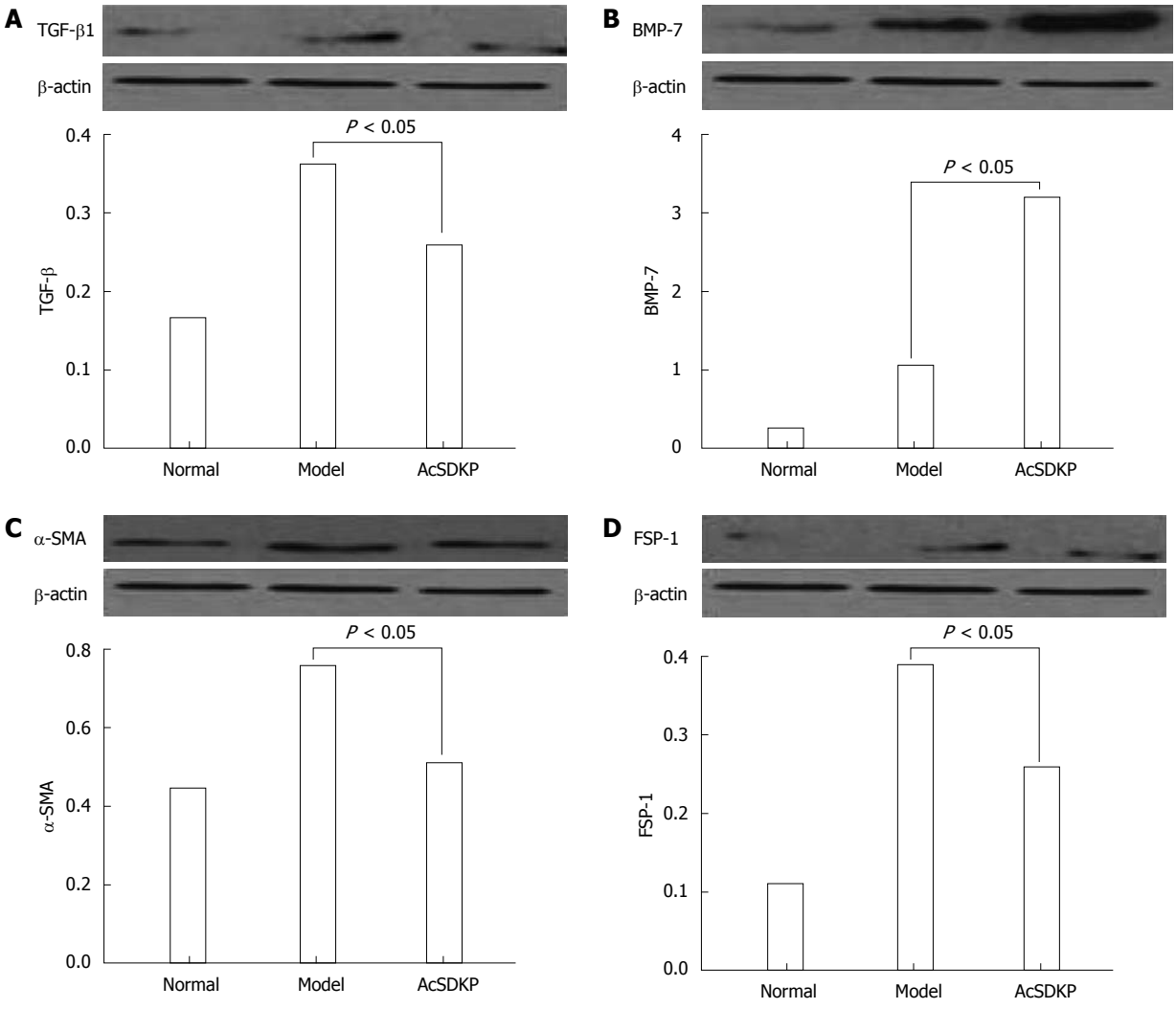


Figure 1 Western blotting and quantitive analysis. A: Transforming growth factor-β1 (TGF-β1); B: Bone morphogenetic protein-7 (BMP-7); C: α-smooth muscle actin positivity (α-SMA); D: Fibroblast specific protein 1 (FSP-1).

RESULTS

Chronic exogenous AcSDKP infusion suppressed profibrogenic TGF-β1 signaling, α-SMA, eibroblast specific protein-1 and bone morphogenetic protein-7 staining and collagen gene expression

When compared to model rats, TGF-β1 was significantly downregulated in AcSDKP-infused BDL-treated rats (Figure 1A). In contrast, bone morphogenetic protein-7 (BMP-7) staining in the liver of BDL-treated rats was increased by AcSDKP (Figure 1B). α-SMA, fibroblast specific protein-1 (FSP-1), collagen I, collagen Ⅲ, tissue inhibitor of metalloproteinase-1 and 2 mRNA all were downregulated by AcSDKP infusion (Figure 1C and D). Collagen Ⅰ, collagen Ⅲ, matrix metalloproteinases-2, tissue inhibitors of metalloproteinase-1 and tissue inhibitors of metalloproteinase-2 mRNA expressions were all significantly downregulated by AcSDKP infusion (2.02 ± 1.10 *vs* 14.16 ± 6.50, 2.02 ± 0.45 *vs* 10.00 ± 3.35, 2.91 ± 0.30 *vs* 7.83 ± 1.10, 4.64 ± 1.25 *vs* 18.52 ± 7.61, 0.46 ± 0.16 *vs* 0.34 ± 0.12, respectively, $P < 0.05$). Matrix metalloproteinase-2 expression was increased in BDL-treated rats but suppressed by AcSDKP.

Chronic exogenous AcSDKP infusion attenuated BDL-induced liver injury, inflammation and fibrosis

BDL caused a remarkable increase in ALT, AST, total bilirubin, and prothrombin time, all of which were reduced by AcSDKP infusion (Table 1). The histological appearance of liver specimens was also improved (Figure 2A-C). Marked collagen accumulation was observed in AcSDKP-infused BDL-treated *vs* model rats, which was attenuated by AcSDKP infusion (Figure 2D-F). The reduction in total collagen was further confirmed by decreased hydroxyproline content. When compared to model rats, hyaluronic acid, ammonia terminal procollagen β peptide and hydroxyproline were all significantly decreased by AcSDKP infusion (127.4 ± 31.8 *vs* 267.2 ± 99.4, 6.9 ± 0.5 *vs* 35.2 ± 4.3, 162.3 ± 42.4 *vs* 398.2 ± 60.4, respectively, $P < 0.05$). Total mast cells decreased in AcSDKP *vs* model BDL-treated rats (Figure 2G-I).

DISCUSSION

Here, we demonstrate that in the liver, chronic exogenous AcSDKP infusion preserves basal levels of AcSDKP and attenuates BDL-induced fibrosis. This is supported by

Table 1 Comparision of liver functions

Group	*n*	ALT (IU/L)	AST (IU/L)	TBIL (μmol/L)	AKP (IU/L)	PT (S)	ALB (g/L)
Normal	10	71.0 ± 32.8	146.0 ± 36.7	1.0 ± 0.8	221.8 ± 96.5	9.3 ± 1.6	38.1 ± 0.9
Model	8	66.9 ± 46.7	472.1 ± 236[a]	56.4 ± 53.9[a]	299.1 ± 37.4[a]	16.6 ± 4.6[a]	29.7 ± 9.5[a]
BDL + AcSDK	8	70.0 ± 34.9	245.7 ± 92.8[c]	6.3 ± 3.3[c]	265.4 ± 77.5	11.8 ± 1.0[c]	34.0 ± 4.9

[a]$P < 0.05$ *vs* normal; [c]$P < 0.05$ *vs* model. BDL: Bile duct ligation; AcSDK: N-acetyl-seryl-aspartyl-lysyl-proline; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; AKP: Alkaline phosphatase; PT: Prothrombin time; ALB: Albumin.

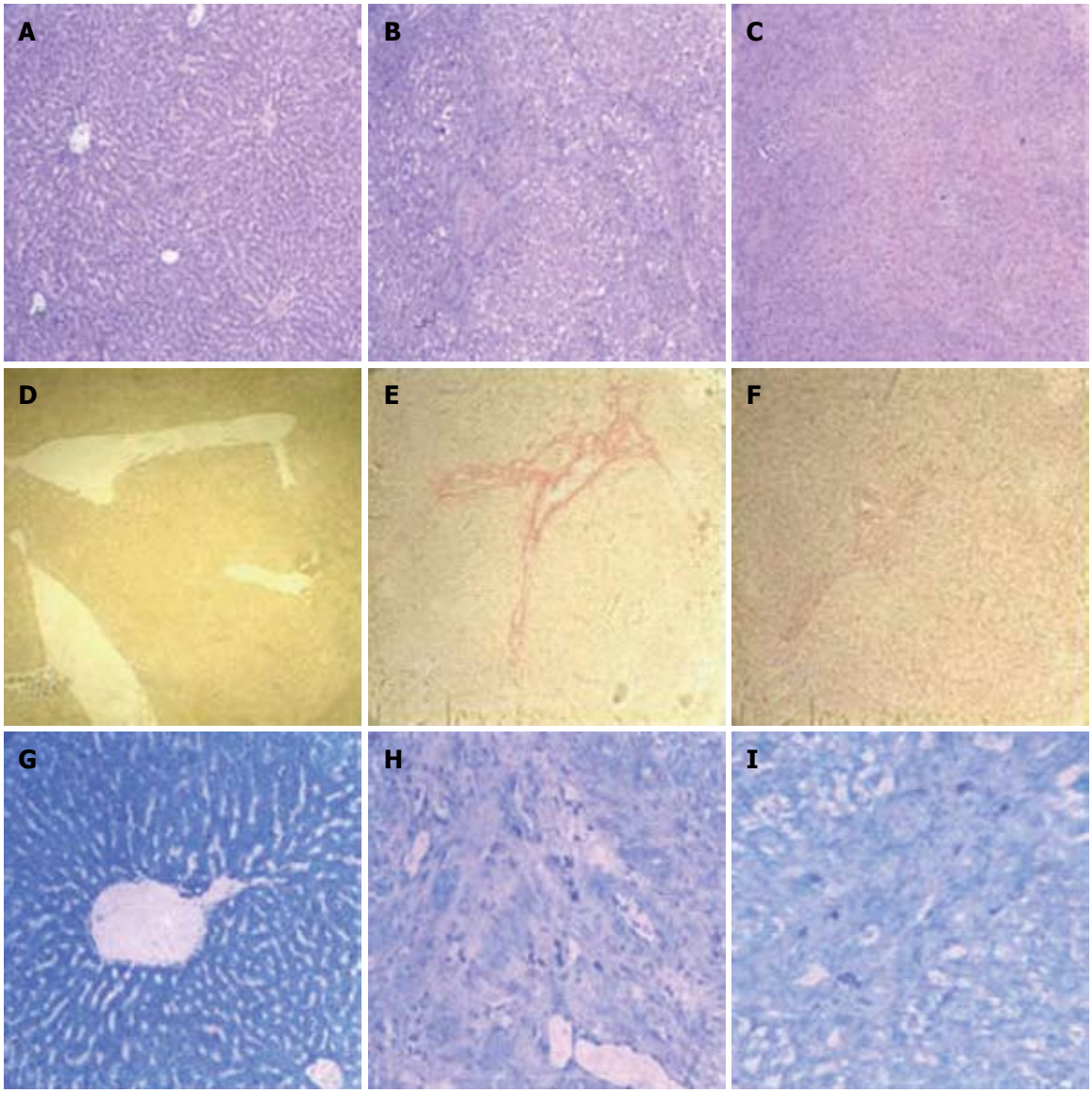


Figure 2 Hematoxylin and eosin, Sirius red, Giemsa staining for liver tissues in each group. A: Normal, hematoxylin and eosin (HE) (× 100); B: Model, HE (× 100); C: N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), HE (× 100); D: Normal, Sirius red (× 100); E: Model, Sirius red (×100); F: AcSDKP, Sirius red (× 100); G: Normal, Giemsa (× 200); H: Model, Giemsa (× 200); I: AcSDKP, Giemsa (× 200).

other studies showing an important role of AcSDKP in preventing heart[19,20] and kidney[21,22] fibrosis at basal concentrations. Our previous studies had also revealed that AcSDKP ameliorated CCl_4-induced liver fibrosis and liver functions in rats.

Attenuation of liver fibrosis by AcSDKP is associated with suppressed inflammation and TGF-β signaling. Our results show that AcSDKP suppressed mast cells infiltration, TGF-β1 signaling and myofibroblasts *in vivo*.

Recent evidence suggests that epithelial-to-mesenchymal transition (EMT) may also contribute to liver fibrogenesis[23]. TGF-β1 is still generally considered to be the main positive regulator of EMT and ECM accumulation[23]. Indeed, our results show that AcSDKP suppressed TGF-β signaling and reduced the EMT markers α-SMA and FSP-1 *in vivo*[24,25]. In addition, AcSDKP increased

BMP-7. BMP-7 counteracts the effects of TGF-β1 and is a prototypical negative regulator of EMT. Nevertheless, a more sophisticated study is required to fully elucidate the possible role of AcSDKP-induced inhibition of EMT in the attenuation of liver fibrosis.

There are some limitations in this study. We did not include a group of control rats infused with AcSDKP primarily because exogenous infusion of AcSDKP restored the peptide levels to control levels and this dose has been shown to have no adverse effects systemically. Secondly, the cellular mechanisms of AcSDKP action were not fully elucidated in our current study. The presence of an AcSDKP receptor on cells has been suggested[26]. We speculate that AcSDKP may directly affect liver cells by binding and activating its receptor on the cell surface, resulting in suppression of certain profibrogenic intracellular signaling pathways. Further studies to clone the receptor or develop specific receptor antagonists will enable full characterization of the cellular mechanisms involved in the antifibrotic effects of AcSDKP *in vivo* and *in vitro*.

In summary, this study shows that chronic exogenous AcSDKP infusion preserves basal levels of AcSDKP and attenuates liver fibrosis induced by BDL in rats. Our study strongly suggests a significant role for AcSDKP in the development of liver fibrosis and potentiates the usefulness of this tetrapeptide in the prevention of this disease. Additional studies are needed to gain further insight into the biological effect of AcSDKP in the liver and further studies are ultimately warranted in the human.

COMMENTS

Background

N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is an endogenous tetrapeptide *in vivo* which has antifibrogenic effects on the heart, the lung and the kidney. The authors' previous studies had revealed that AcSDKP ameliorated carbon tetrachloride-induced liver fibrosis and liver functions in the rat liver.

Research frontiers

Originally described as a natural inhibitor of hematopoietic stem cell proliferation, AcSDKP is now recognized as a critical negative regulator for extracellular matrix accumulation in organs under both physiological and pathological conditions.

Innovations and breakthroughs

This is the first study to investigate the preventive effect of endogenous AcSDKP in bile duct ligation (BDL)-induced fibrosis in the rat liver and the potential mechanisms involved were also examined. The results strongly suggest a significant role for AcSDKP in the development of liver fibrosis and potentiates the usefulness of this tetrapeptide in the prevention of this disease.

Applications

Preservation of AcSDKP may be a useful therapeutic approach in the management of liver fibrosis.

Peer review

This is a good descriptive study in which authors analyze the preventive effect of AcSDKP on BDL-induced liver fibrosis in rats. The results are interesting and suggest that infusion of exogenous AcSDKP attenuated BDL-induced fibrosis in the rat liver. Preservation of AcSDKP may be a useful therapeutic approach in the management of liver fibrosis.

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BRIEF ARTICLE

Minilaparotomy to rectal cancer has higher overall survival rate and earlier short-term recovery

Xiao-Dong Wang, Ming-Jun Huang, Chuan-Hua Yang, Ka Li, Li Li

Xiao-Dong Wang, Ming-Jun Huang, Chuan-Hua Yang, Ka Li, Li Li, Gastrointestinal Surgery Center, West China Hospital in Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Wang XD and Li L designed the study; Wang XD, Yang CH and Li L performed the clinic works in the study (operations and peri-operative management); Wang XD, Huang MJ and Li K collected information and research data; Wang XD and Huang MJ analyzed the data and wrote the manuscript.

Correspondence to: Dr. Li Li, Gastrointestinal Surgery Center, West China Hospital in Sichuan University, Chengdu 610041, Sichuan Province, China. drlili116@126.com
Telephone: +86-28-85422481 Fax: +86-28-85422481
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Abstract

AIM: To report our experience using mini-laparotomy for the resection of rectal cancer using the total mesorectal excision (TME) technique.

METHODS: Consecutive patients with rectal cancer who underwent anal-colorectal surgery at the authors' hospital between March 2001 and June 2009 were included. In total, 1415 patients were included in the study. The cases were divided into two surgical procedure groups (traditional open laparotomy or mini-laparotomy). The mini-laparotomy group was defined as having an incision length ≤ 12 cm. Every patient underwent the TME technique with a standard operation performed by the same clinical team. The multimodal preoperative evaluation system and postoperative fast track were used. To assess the short-term outcomes, data on the postoperative complications and recovery functions of these cases were collected and analysed. The study included a plan for patient follow-up, to obtain the long-term outcomes related to 5-year survival and local recurrence.

RESULTS: The mini-laparotomy group had 410 patients, and 1015 cases underwent traditional laparotomy. There were no differences in baseline characteristics between the two surgical procedure groups. The overall 5-year survival rate was not different between the mini-laparotomy and traditional laparotomy groups (80.6% *vs* 79.4%, $P = 0.333$), nor was the 5-year local recurrence (1.4% *vs* 1.5%, $P = 0.544$). However, 1-year mortality was decreased in the mini-laparotomy group compared with the traditional laparotomy group (0% *vs* 4.2%, $P < 0.0001$). Overall 1-year survival rates were 100% for Stage Ⅰ, 98.4% for Stage Ⅱ, 97.1% for Stage Ⅲ, and 86.6% for Stage Ⅳ. Local recurrence did not differ between the surgical groups at 1 or 5 years. Local recurrence at 1 year was 0.5% (2 cases) for mini-laparotomy and 0.5% (5 cases) for traditional laparotomy ($P = 0.670$). Local recurrence at 5 years was 1.5% (6 cases) for mini-laparotomy and 1.4% (14 cases) for traditional laparotomy ($P = 0.544$). Days to first ambulation (3.2 ± 0.8 d *vs* 3.9 ± 2.3 d, $P = 0.000$) and passing of gas (3.5 ± 1.1 d *vs* 4.3 ± 1.8 d, $P = 0.000$), length of hospital stay (6.4 ± 1.5 d *vs* 9.7 ± 2.2 d, $P = 0.000$), anastomotic leakage (0.5% *vs* 4.8%, $P = 0.000$), and intestinal obstruction (2.2% *vs* 7.3%, $P = 0.000$) were decreased in the mini-laparotomy group compared with the traditional laparotomy group. The results for other postoperative recovery function indicators, such as days to oral feeding and defecation, were similar, as were the results for immediate postoperative complications, including the physiologic and operative severity score for the enumeration of mortality and morbidity score.

CONCLUSION: Mini-laparotomy, as conducted in a single-centre series with experienced TME surgeons, is a safe and effective new approach for minimally invasive rectal cancer surgery. Further evaluation is required to evaluate the use of this approach in a larger patient sample and by other surgical teams.

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Key words: Rectal neoplasm; Mini-laparotomy; Survival; Total mesorectal excision

Peer reviewers: San-Jun Cai, MD, Professor, Director, Department of Colorectal Surgery, Cancer Hospital, Fudan University, 270 Dong An Road, Shanghai 200032, China; Francis Seow-Choen, MBBS, FRCSEd, FAMS, Professor, Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, 3 Mt Elizabeth Medical Centre No. 09-10, Singapore 228510, Singapore

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INTRODUCTION

Laparoscopic surgery has become popular throughout the world[1-3]. This surgical method enhances early postoperative recovery with less pain, less analgesic use, an earlier return of gastrointestinal function, and fewer wound and pulmonary complications. Total mesorectal excision (TME) is acknowledged worldwide as the preferred technique for surgical resection of rectal cancer[4-6]. Multiple studies have reported marked reductions in local recurrence with the TME technique, including single-centre, multiple-centre, and population studies[7,8]. The laparoscopic approach to TME resection of rectal cancer is currently being evaluated in multicentre randomised trials[9-12].

A less-recognised surgical technique aimed at improving postoperative recovery is mini-laparotomy. With this technique, surgical dissection is performed under direct vision, as in open surgery; laparoscopic equipment and training is not required. Early experience with mini-laparotomy has been reported from a few medical centres in case series of colon and rectal resection[13-15]. Mini-laparotomy has been developed as a techniques based on the advanced recognition of more information about pelvic anatomy and the dissection of subtle perirectal structures in laparotomy[16,17].

In view of these circumstances, our surgical centre has performed mini-laparotomies for rectal cancer for approximately 8 years. The aim of this study is to report our experience using mini-laparotomy for the resection of rectal cancer using the TME technique. Furthermore, we aim to compare the oncologic findings and the postoperative recovery indexes of mini-laparotomy and traditional laparotomy, thus providing more evidence to help surgeons select an operating procedure.

MATERIALS AND METHODS

Included cases

This study is registered as an International Clinical Trial (ChiCTR-TRC-09000618) to compare TME resection of rectal cancer using traditional open laparotomy *vs* mini-laparotomy. This is a retrospective analysis of consecutive patients with rectal cancer observed at the Anal-colorectal Surgery Ward in West China Hospital of Sichuan University between March 2001 and June 2009. The inclusion criteria were as follows: (1) diagnosis of rectal cancer; (2) no previous history of lower abdominal operations or pelvic operations; (3) possibility of curative resection; and (4) intestinal continuity was restored by anastomosis. The exclusion criteria were (1) curative resection was not achieved; (2) resections without anastomosis (APR and Hartmann); and (3) actively exciting the study. All of the enrolled patients provided informed consent, which included information about (1) the different kinds of treatment available for their cancer; (2) the benefit of different operation procedures; and (3) their doctor's recommendation. Ultimately, the choice of surgical technique was left to the patient. The database from the anal-colorectal surgery of West China Hospital in Sichuan University provided the research data[18]. If any data required for the study were missing, the patient was excluded. Most of the patients who were excluded for this reason were missing data related to pathology and surgical baselines; 5-year survival and local recurrence; and the first time of aerofluxus, defecation, ambulation, oral feeding during the recovery phase. Ultimately, 1415 patients were included in the study.

A multimodal preoperative evaluation system was used to assess the preoperative clinical cancer stage[19]. Clinical Stages Ⅲ and Ⅳ patients were treated with neoadjuvant and adjuvant chemotherapy consisting of FOLFOX-4 (Oxaliplatin 85 mg/m^2 ivgtt 2 h, 1 d; LV 200 mg/m^2 ivgtt 2 h, 1-2 d; 5-Fu 400 mg/m^2 *iv*, 1-2 d). Perioperative radiation is not used at our centre.

Surgery was performed by traditional open laparotomy as the standard procedure. Mini-laparotomy was performed on an ad-hoc basis, with increasing frequency in the latter years of this study. No patient within the mini-laparotomy group was converted to a traditional laparotomy.

Short-term perioperative data were obtained in all cases. Long-term follow-up data were available from 7 to 103 mo. The follow-up methods used included telephone follow-up, outpatient department follow-up and follow-up letters. Follow-up data were obtained in 96.3% of cases (1362/1415).

Operation and clinical management procedures

TME and pelvic autonomic nerve preservation were performed in all cases in accordance with the Colorectal Surgery Guideline of West China Hospital of Sichuan University[20]. The surgery and perioperative management were performed by the same clinical team for both the traditional open laparotomy and mini-laparotomy groups.

A vertical incision was used for all cases. The traditional laparotomy incision extended from the pubis to above the umbilicus with a length of 13 to 18 cm, as in Figure 1. The mini-laparotomy incision extended from

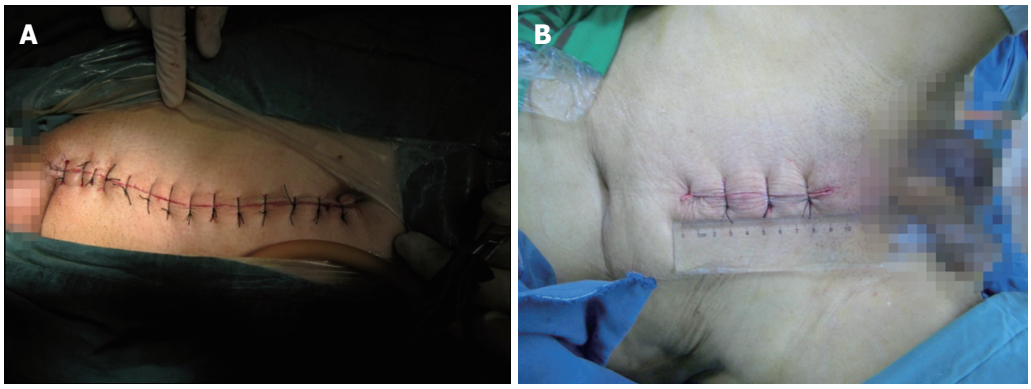


Figure 1 Mini-laparotomy (A) and traditional laparotomy (B).

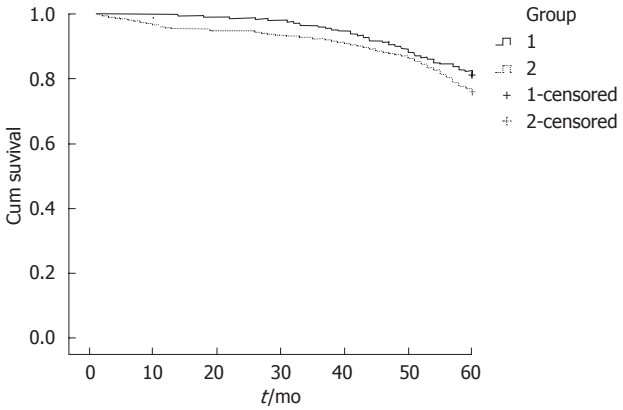


Figure 2 Survival rates of the mini-laparotomy (1) and traditional laparotomy groups (2).

the pubis for a length of 12 cm or less. For the traditional laparotomy, a fixed self-retaining retractor was used on the incision, and a moveable curved retractor was used for dynamic exposure during dissection. In mini-laparotomy, two curved retractors were used in dynamic exposure during dissection without a fixed abdominal wound retractor. Dissection was performed using electrocautery and an ultrasonic knife in both open laparotomy and mini-laparotomy. In both mini-laparotomy and traditional laparotomy, the splenic flexure was not mobilised. The superior rectal artery was ligated just below the bifurcation of the inferior mesenteric artery with clearing of the superior rectal artery lymph nodes.

A post-operative fast-track protocol was used[21,22] with early ambulation. Discharge criteria included eating a normal diet, normal ambulation, no fever, and no post-operative complications.

Definition of outcome

The primary outcome variables are 5-year survival and local recurrence. Local recurrence was classified as intro-/peri-anastomotic and pelvic recurrence. Secondary outcomes are immediate postoperative complications, including physiologic and operative severity score for the enumeration of mortality and morbidity (POSSUM) score[23], and recovery functions (the time of the first aerofluxus, defecation, ambulation, and oral feeding during the recovery phase). In addition, the patients' postoperative complications were recorded as important information about secondary outcomes, which included gastric retention, incision infection, pulmonary infection, anastomosis leakage, and intestinal obstruction.

Statistical analysis

The mini-laparotomy and traditional laparotomy data were compared and analysed with *t* tests, and the count data used the χ^2 test or Fisher's exact probability test. Local recurrence and overall survival were assessed using Kaplan-Meier survival curve analysis. The data are expressed as means ± SD. The significance level was 0.05. Statistical tests were performed using SPSS 15.0.

RESULTS

Baseline characteristics

The characteristics of the 1415 included patients (410 mini-laparotomy cases and 1005 traditional laparotomy cases) are presented in Table 1. There were no significant difference in the baseline data for the two groups ($P > 0.05$). The surgical and pathological findings are presented in Table 2, and there were no significant differences between the two surgical groups ($P > 0.05$).

Overall survival and local recurrence

Overall 5-year survival did not differ between the mini-laparotomy and traditional laparotomy groups (80.6% *vs* 79.4%, $P = 0.333$; Figure 2). The 5-year survival rates for the different clinical stages were also similar. One-year mortality was decreased in the mini-laparotomy group compared to the traditional laparotomy group (0% *vs* 4.2%, $P < 0.0001$). The overall 1-year survival rates in the traditional group were 100% for Stage Ⅰ, 98.4% for Stage Ⅱ, 97.1% for Stage Ⅲ, and 86.6% for Stage Ⅳ. Local recurrence did note differ between surgical groups at 1 or 5 years. Local recurrence at 1 year was 0.5% (2 cases) for the mini-laparotomy group and 0.5% (5 cases) for the traditional laparotomy group ($P = 0.670$). Local recurrence at 5 years was 1.5% (6 cases) for the mini-laparotomy group and 1.4% (14 cases) for the traditional laparotomy group ($P = 0.544$).

Table 1 Baseline characteristics of the mini-laparotomy and traditional laparotomy groups

	Minilaparotomy (n = 410)	Traditional laparotomy (n = 1005)	P value
Gender			0.125
Male	273 (66.6)	635 (63.2)	
Female	137 (33.4)	370 (36.8)	
Age (yr)	61.2 ± 12.1	57.8 ± 12.4	0.385
BMI (kg/m^2)	21.3 ± 3.0	22.0 ± 2.9	0.331
Distance to dentate line (cm)	8.2 ± 3.2	7.2 ± 4.0	0.118

BMI: Body mass index.

Table 2 Surgical and pathological findings for the mini-laparotomy and traditional laparotomy groups

	Minilaparotomy	Traditional laparotomy	P value
TNM stage			0.838
Stage Ⅰ	47 (11.5)	119 (11.8)	
Stage Ⅱ	127 (31.0)	320 (31.8)	
Stage Ⅲ	151 (36.8)	379 (37.7)	
Stage Ⅳ	85 (20.7)	187 (18.6)	
Differentiation			0.579
Good	105 (25.6)	240 (23.9)	
Moderate	172 (42.0)	411 (40.9)	
Poor	133 (32.4)	354 (35.2)	
Histologic types			0.277
Adenocarcinoma	337	811	
Mucinous adenocacinoma	69	164	
Squamous carcinoma	4	30	
Operation types			0.640
High anterior resection	20 (4.9)	38 (3.8)	
Low anterior resection	124 (30.2)	284 (28.3)	
Ultralow anterior resection	189 (46.1)	482 (48.0)	
Colo-anal anastomosis	77 (18.8)	201 (20.0)	
Volume of bleeding (mL)	78.5 ± 30.0	80.8 ± 28.5	0.940
Operation time (min)	115.5 ± 35.8	114.6 ± 33.4	0.217
Lymph node counts	12.4	12.7	0.796
Proximal margin of distance (cm)	3.5	3.3	0.105
Distal margin of distance (cm)	7.0	6.9	0.780

TNM: Tumor-node-metastasis. Data are presented as n (%) or mean ± SD.

Short-term recovery and postoperative complications

The data regarding postoperative recovery functions (time of first aerofluxus, defecation, ambulation and oral feeding), length of hospital stay, and immediate postoperative complications, including POSSUM score, are shown in Table 3. Days to first ambulation and aerofluxus and hospital length of stay were reduced for the mini-laparotomy group compared with the traditional laparotomy group. Days to tolerating full oral diet and first defecation were similar between surgical groups. POSSUM scores predicting mortality and morbidity were not different between the surgical groups.

Gastric retention and wound and pulmonary infection were not different between the surgical groups. Anastomotic leakage and intestinal obstruction were decreased in the mini-laparotomy group compared with the traditional laparotomy group. Other postoperative complications,

Table 3 Postoperative recovery of the two groups

	Minilaparotomy group	Traditional laparotomy group	P value
Recovery			
Aerofluxus (d)	3.5 ± 1.1	4.3 ± 1.8	0.000
Oral feeding (d)	4.1 ± 1.2	4.6 ± 1.2	0.628
Defecation (d)	5.0 ± 1.4	5.4 ± 1.5	0.370
Ambulation (d)	3.2 ± 0.8	3.9 ± 2.3	0.000
Hospital stay (d)	6.4 ± 1.5	9.7 ± 2.2	0.000
POSSUM scores (%)			
Predictive mortality	28.1	26.8	0.738
Predictive morbidity	5.3	5.0	0.844
Complications			
Gastric retention	8 (2.0)	18 (1.8)	0.494
Incision infection	6 (1.5)	15 (1.5)	0.592
Pulmonary infection	5 (1.2)	14 (1.4)	0.513
Anastomosis leakage	2 (0.5)	48 (4.8)	0.000
Intestinal obstruction	9 (2.2)	73 (7.3)	0.000
Other	13 (3.2)	35 (3.5)	0.456

POSSUM: Physiologic and operative severity score for the enumeration of mortality and morbidity.

Table 4 Colorectal cancer surgery study results regarding local recurrence and survival rates (%)

Another preference	Local recurrence	Survival rate
Our study	1.4	79.7
Andreoni *et al*[30]	8.2	71.0
Law *et al*[31]	9.6	66.5
Jung *et al*[7]	8.0	62.0

including urinary retention (2 cases *vs* 6 cases), urinary tract infection (0 case *vs* 2 cases), anastomotic bleeding (1 case *vs* 2 cases), intra-abdominal haemorrhage (0 case *vs* 1 case), wound dehiscence (1 case *vs* 2 cases), sexual dysfunction (1 case *vs* 3 cases), deep vein thrombosis (0 case *vs* 0 case), cardiocerebral vascular accident (0 case *vs* 0 case), psychosis (2 cases *vs* 4 cases), liver dysfunction (1 case *vs* 2 cases), and unknown fever (5 cases *vs* 13 cases) also did not differ between the two surgical groups.

DISCUSSION

The overall 5-year survival and local recurrence rates were not different between the mini-laparotomy and traditional laparotomy groups. The local recurrence and survival rates for mini-laparotomy (1.4% and 79.7%) compare favourably to those reported for traditional laparotomy, as shown in Table 4.

In our study, the results confirmed that the mini-laparotomy and traditional laparotomy groups had similar overall 5-year survival and local recurrence rates, but the minilaparotomy group experienced faster postoperative recovery and fewer complications. It may be that mini-laparotomy surgery is the safer and more effective operation for rectal cancer.

An improved understanding of pelvic anatomy has enabled the switch to a mini-laparotomy approach to rec-

tal cancer resection. Whereas others have reported on the use of mini-laparotomy mostly for colon cancer, we are among the first to report on the use of mini-laparotomy for the resection of rectal cancer[8,9,12]. There is a learning curve for the mini-laparotomy approach. However, we speculate that the learning curve is lower than that of laparoscopic proctectomy because standard instrumentation, direct vision and tactile feedback are maintained for mini-laparotomy, but not for laparoscopy. We have used mini-laparotomy for rectal resection in Chinese patients with a body mass index (BMI) of 21.3 kg/m^2 as the Chinese population is generally thinner than North American and European populations[24]; however, we also treat patients with BMIs > 27 using mini-laparotomy. Additionally, although splenic flexure mobilisation was not required for any patient in this sample, it is likely that the surgeon would be disadvantaged by the suprapubic mini-laparotomy approach if splenic flexure mobilisation was required to perform a low tension-free anastomosis. Mini-laparotomy was not associated with an increase in operative blood loss or operation time. In fact, mini-laparotomy was associated with the successful resection of all levels of rectal cancer.

Like laparoscopic colorectal resection[25], mini-laparotomy showed advantages in decreasing the postoperative length of hospital stay and was associated with shorter times to ambulation and aerofluxus[1,11]. Mini-laparotomy was associated with significantly decreased anastomotic leaking, a finding for which we have no ready explanation other than increased experience over the time of the study. The anastomotic leak rate of 0.5% compares favourably to that reported in other studies[26-28]. In addition, mini-laparotomy was associated with decreased postoperative intestinal obstruction, which may have resulted from less peritoneal manipulation and a shorter incision length. A Japanese study suggested that the mini-laparotomy approach in colorectal cancer would result in a reduced inflammatory response[29]. Furthermore, mortality at 1 year was significantly increased in the traditional laparotomy group compared with the mini-laparotomy group.

Although postoperative management was intended to be similar for mini-laparotomy and traditional laparotomy patients, as indicated by the time before achieving a full oral diet and defecation, traditional laparotomy patients were slower to ambulate. Bias in favour of the mini-laparotomy group is likely, as this procedure has gained favour in our hands with our increasing experience over the time of this study. A randomised study design is indicated to minimise this bias. The study was a single-centre series with experienced TME surgeons and a patient sample with a relatively small BMI. Because our hospital is a medical centre in southwestern China, our patients are primarily advanced cancer cases, and we have more experienced surgeons and more advanced surgical instruments than other small-to-medium sized local hospitals. Consequently, the study and results had an obvious selection bias. Further evaluation is required to evaluate the use of this approach in a larger patient sample and by other surgical teams, and our conclusions are not definitive.

In conclusion, we have shown that mini-laparotomy is a safe and effective new approach for minimally invasive rectal cancer surgery. This was a single-centre series with experienced TME surgeons and a patient sample with a relatively low BMI. Further evaluation is required to evaluate the use of this approach in a larger patient sample and by other surgical teams.

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COMMENTS

Background

Total mesorectal excision (TME) is acknowledged worldwide as the preferred technique for surgical resection of rectal cancer. The laparoscopic approach to TME resection of rectal cancer is currently being evaluated in multicentre randomised trials. A less-recognised surgical technique aimed at improving postoperative recovery is mini-laparotomy. With this technique, surgical dissection is performed under direct vision, as in open surgery; laparoscopic equipment and training is not required. Early experience with mini-laparotomy has been reported from a few medical centres in case series of colon and rectal resection. Mini-laparotomy has been developed as a techniques based on the advanced recognition of more information about pelvic anatomy and the dissection of subtle perirectal structures in laparotomy.

Research frontiers

An improved understanding of pelvic anatomy has enabled the switch to a mini-laparotomy approach to rectal cancer resection. Other studies have reported on the use of mini-laparotomy mostly for colon cancer, but for rectal cancer, it is still in blank. Like laparoscopic colorectal resection, mini-laparotomy showed advantages in decreasing the postoperative length of hospital stay and was associated with shorter times to ambulation and aerofluxus in past studies

Innovations and breakthroughs

Whereas others have reported on the use of mini-laparotomy mostly for colon cancer, the authors are among the first to report on the use of mini-laparotomy for the resection of rectal cancer. There is a learning curve for the mini-laparotomy approach. However, the authors speculate that the learning curve is lower than that of laparoscopic proctectomy because standard instrumentation, direct vision and tactile feedback are maintained for mini-laparotomy, but not for laparoscopy. Mini-laparotomy was associated with significantly decreased anastomotic leaking, a finding for which the authors have no ready explanation other than increased experience over the time of the study. The anastomotic leak rate of 0.5% compares favourably to that reported in other studies. In addition, mini-laparotomy was associated with decreased postoperative intestinal obstruction, which may have resulted from less peritoneal manipulation and a shorter incision length. Furthermore, mortality at 1 year was significantly increased in the traditional laparotomy group compared with the mini-laparotomy group.

Applications

The study results suggest that mini-laparotomy is a safe and effective new approach for minimally invasive rectal cancer surgery.

Terminology

Minilaparotomy is minimal invasive laparotomy for abdominal operations. It is different from laparoscopic operations, that minilaparotomy will have only one mini-incision, but not other holes from laparoscopic way. Minilaparotomy would be more better outcomes than traditional laparotomy.

Peer review

This is a good descriptive study conducted in a single-centre series with experienced TME surgeons, and mini-laparotomy is proved a safe and effective new approach for minimally invasive rectal cancer surgery. Minilaparotomy will be the future choice for rectal cancer care.

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BRIEF ARTICLE

Evaluation of a new method for placing nasojejunal feeding tubes

Hua Qin, Xiao-Yun Lu, Qiu Zhao, De-Min Li, Pei-Yuan Li, Mei Liu, Qi Zhou, Liang Zhu, Hui-Fang Pang, Hui-Zhen Zhao

Hua Qin, Xiao-Yun Lu, Qiu Zhao, De-Min Li, Pei-Yuan Li, Mei Liu, Qi Zhou, Liang Zhu, Hui-Fang Pang, Hui-Zhen Zhao, Department of Gastroenterology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

Author contributions: Qin H and Lu XY contributed equally to this work; Qin H, Lu XY and Zhao Q designed the research; Qin H, Zhao Q, Li DM, Li PY, Liu M and Zhou Q performed the research; Zhu L and Pang HF provided new reagents/analytic tools; Lu XY and Zhao HZ analyzed the data; and Qin H and Lu XY wrote the paper.

Correspondence to: Qiu Zhao, MD, Professor of Medicine, Department of Gastroenterology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, No.1095 Jiefang Avenue, Wuhan 430030, Hubei Province, China. zhaoqiu@medmail.com.cn

Telephone: +86-27-83663334 Fax: +86-27-83663661
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Abstract

AIM: To compare fluoroscopic, endoscopic and guide wire assistance with ultraslim gastroscopy for placement of nasojejunal feeding tubes.

METHODS: The information regarding nasojejunal tube placement procedures was retrieved using the gastrointestinal tract database at Tongji Hospital affiliated to Tongji Medical College. Records from 81 patients who underwent nasojejunal tubes placement by different techniques between 2004 and 2011 were reviewed for procedure success and tube-related outcomes.

RESULTS: Nasojejunal feeding tubes were successfully placed in 78 (96.3%) of 81 patients. The success rate by fluoroscopy was 92% (23 of 25), by endoscopic technique 96.3% (26 of 27), and by guide wire assistance (whether *via* transnasal or transoral insertion) 100% (23/23, 6/6). The average time for successful placement was 14.9 ± 2.9 min for fluoroscopic placement, 14.8 ± 4.9 min for endoscopic placement, 11.1 ± 2.2 min for guide wire assistance with transnasal gastroscopic placement, and 14.7 ± 1.2 min for transoral gastroscopic placement. Statistically, the duration for the third method was significantly different ($P < 0.05$) compared with the other three methods. Transnasal placement over a guidewire was significantly faster ($P < 0.05$) than any of the other approaches.

CONCLUSION: Guide wire assistance with transnasal insertion of nasojejunal feeding tubes represents a safe, quick and effective method for providing enteral nutrition.

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Key words: Enteral nutrition; Nasojejunal feeding tube; Guide wire assistance; Fluoroscopy; Endoscopy

Peer reviewer: Dr. Laura E Matarese, Department of Gastroenterology, Hepatology and Nutrition, East Carolina Univeristy, PCMH MA 338, Greenville, NC 27834, United States

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INTRODUCTION

Enteral nutrition (EN) not only provides energy support as with parenteral nutrition, but also maintains the functional intestinal barrier, significantly reducing the incidence of infection and organ failure, shortens hospital

stays, and lowers treatment costs[1-4]. EN has therefore become an important nutritional therapy[5]. A nasogastric (NG) tube is often associated with some problems of large gastric residual volumes, reflux and vomiting, while a nasojejunal (NJ) tube and prokinetic agents are useful for circumventing the problems associated with upper gastrointestinal intolerance of NG feeding[6]. NJ feeding tubes positioned beyond the ligament of Treitz's have been shown to allow early attainment of caloric needs and a reduction in tube-feeding aspiration events in patients with gastric feeding intolerance[7,8]. There are presently several methods for placement of NJ feeding tubes[9-11]. Previously, we would use fluoroscopic placement under direct endoscopic visualization instead of using NJ tubes.

Recently, we have applied an ultrathin transnasal endoscope which afforded us a higher success rate and a shortened procedure time. Herein we evaluated the usefulness and safety of this new method compared with the other two traditional methods.

MATERIALS AND METHODS

Patients

This is a retrospective study involving the patients who were treated with enteral feeding from January 2004 through September 2011 at our hospital. Written informed consent was obtained from all patients or their representatives. The Ethics Committee of Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, approved the study protocol. All subjects were $\geqslant$ 18 years of age. The technique, success rate, procedure duration, and complications were recorded for each patient. Patient sex, age and diseases were also recorded. According to the placement methods, the patients were divided into three groups as described below. A 130-cm long polyurethane nasoenteral feeding tube with a front-end opening (Flocare, Nutricia, Netherlands) was used in each case. All NG tubes were removed before the start of the procedure, as they might have interfered with placement of both the feeding tube and endoscope.

Fluoroscopic technique

The feeding tube was placed by one skilled doctor. Some gastroenterologists were also involved in the fluoroscopy. Additional sedation was not required for fluoroscopic tube placement. A portable C-arm fluoroscope was positioned over the supine patient's abdomen. The timing of the procedure began when the feeding tube entered the nares. When the tube was advanced to 50-55 cm, its position was evaluated by intermittent fluoroscopy. The operators rotated the feeding tube to facilitate passage to the pylorus. Fluoroscopy was used intermittently or continuously as needed. When the tip of the feeding tube was beyond the pylorus, it was gently advanced as far as possible. Placement of the tube beyond the third portion of the duodenum was preferred. Finally, a fluoroscopic print was obtained after 10-15 mL of meglumine diatrizoate was injected into the feeding tube.

Endoscopic technique

All feeding tubes were placed at the Endoscopy Center by one skilled endoscopist. The posterior oropharynx was anesthetized with topical 4% Xylocaine. The timing of the procedure began when the feeding tube entered the nares. The lubricated feeding tube was inserted into the stomach and advanced until resistance was encountered (usually 55-60 cm), and the wire stylet was left in place. At this point, a standard forward-viewing endoscope (Olympus GIF 240 or 260, Olympus Corporation, New York, NY, United States) was placed into the esophagus and then the stomach. The stomach was insufflated with air, and the feeding tube usually traveled along the greater curvature of the stomach. The feeding tube was advanced to the nasopharynx. The distal 10-20 cm of the feeding tube was grasped by the biopsy forceps, and then the tip of the catheter was directed into the pylorus under endoscopic visualization. The feeding tube was then advanced at the nasopharynx, its distal 10-20 cm grasped with biopsy forceps and the tip directed through the pylorus under direct vision. When the feeding tube was observed in a good position, the endoscope was carefully withdrawn with the feeding tube secured by the forceps, which was advanced along with the withdrawal of the endoscope. When the forceps could no longer advance, the endoscope movement was stopped and the forceps was gently pulled back to the end of the lens. Then the forceps was again used to grasp the feeding tube and the above process was repeated until the endoscope was removed completely from the throat. The wire stylet was removed and a fluoroscopic print was obtained after 10-15 mL meglumine diatrizoate was injected into the feeding tube to confirm placement of the feeding tube into the second or third portion of the duodenum.

Guide wire technique

Topical Xylocaine was sprayed into the nose and retropharynx in conscious patients. The tip of the ultraslim transnasal endoscope with an outer diameter of 5.0 mm (Olympus XP-260N, Olympus Corporation, New York city, NY) was then passed under direct vision into one of the nasal passages. Extreme care was taken to avoid traumatizing the mucous membranes. After the endoscope arrived at the third portion of the duodenum, a 260-cm long guide wire with a soft tip (Zebra Exchange Guidewire, Boston Scientific, United States) was inserted along the endoscopic biopsy channel. Using a pull-push technique, the endoscope was slowly withdrawn while the wire was simultaneously threaded forward, so that the wire stayed in a fixed position in the intestine. Before exiting the stomach, the path made by the wire was studied and adjusted to ensure that there were no coils or loops within the gastric body. After withdrawal of the endoscope, an open-ended feeding tube was lubricated and passed over the guide wire, ensuring that the wire remained taut and

Table 1 Patient characteristics

Patient characteristics	Fluoroscopic placement	Endoscopic placement	Guide wire placement
Age (yr), mean ± SD	54.4 ± 9.9	55.8 ± 9.7	56.2 ± 9.5
Gender			
Men	15	14	16
Women	10	13	13
Primary diagnoses			
Pancreatitis	25	14	8
Postoperative gastric cancer	0	6	10
Postoperative esophageal cancer	0	4	6
Abdominal injury	0	1	2
Pancreatic cancer after Whipple surgery	0	1	1
Thoracic esophageal fistula	0	1	1
Gastric perforation	0	0	1

in place. Care was taken not to over-advance the tubes because this often results in coiling in the stomach and loss of duodenal access. Finally, using the adjacent naris, the endoscope was reintroduced into the proximal stomach to check final placement. In most cases, the hub of the wider gastric aspiration tube was too short and had to be advanced gently into the antrum visually, making sure that the tube remained straight along the greater curvature and that the jejunal extension slid further through the pylorus. With an assistant securing the feeding tube to prevent displacement, the endoscope was then eased back into the apex of the body to check the final position before exiting the esophagus. Transnasal endoscopy was not feasible in patients with congestion or stenosis of the nasal passageway. Conventional per-oral endoscopy was used to place the guide wire, which consequently ended up emerging from the mouth. The wire was then redirected through the nose by nasopharyngo-oral cannulation with a small 2-mm internal diameter flexible tube, allowing final placement of the NJ feeding tube.

Statistical analysis

All data were presented as the mean ± SD. The SPSS 15.0 software package (SPSS, Inc., United States) was used for all statistical analyses. Differences between and among outcome groups were determined using the χ^2 test. Significance was determined at P <0.05.

RESULTS

Patient characteristics

Demographic data of the included patients are shown in Table 1. The mean age was 55.5 years (range: 24-70 years). There were 45 men and 36 women. Common primary diagnoses were pancreatitis, postoperative gastric cancer, postoperative esophageal cancer, abdominal injury, pancreatic cancer after Whipple surgery, thoracic esophageal fistula and gastric perforation. All patients demonstrated either high gastric residuals on attempted NG feeding or a physiologic requirement for postduodenal enteral feedings (i.e., pancreatitis), or they were believed to be at high risk for gastric aspiration.

Table 2 Outcome data of the patients

Variables	Fluoroscopic placement	Endoscopic placement	Guide wire placement	
			Transnasal	Transoral
Time to complete procedure (min)	14.9 ± 5.8	14.8 ± 4.9	11.1 ± 2.2[a]	14.7 ± 1.2
Successful placement	23/25 (92)	26/27 (96.3)	23/23 (100)	6/6 (100)
Complications	0/25 (0)	4/27 (14.8)	0/23 (0)	0/6 (0)

Data are presented as mean ± SD or *n/N* (%). [a]P < 0.05 *vs* the other three groups.

Patient outcomes

Outcome data of the patients are shown in Table 2. NJ feeding tubes were successfully placed in 78 of 81 (96.3%) patients. The success rate by fluoroscopy was 92% (23 of 25), by endoscopic technique was 96.3% (26 of 27), and by guide wire was 100% with either transnasal endoscopy or transoral endoscopy. Significant differences between the guide wire assistance with transnasal ultraslim endoscopy and the other three groups were noted in placement duration, whereas there were no significant differences among the other three treatment groups. No significant differences among all the groups were noted in the success or complication rate. No complications were reported from fluoroscopic placement. There were four instances of epistaxis related to replacement of the NG tube after endoscopic placement. All cases of epistaxis resolved without intervention. There was no death related to either procedure.

DISCUSSION

It is well known that malnutrition of critically ill patients is associated with poorer clinical outcomes, and early, sufficient nutritional support can significantly improve the outcomes of the patients[12-16]. EN support is indicated for patients who are unable to take foods orally but have normal intestinal function[17,18], such as those with severe acute pancreatitis, cerebrovascular accidents, traumatic brain injury, *etc*. EN can be delivered through NG tube or NJ tube. The complications of upper gastrointestinal intolerance to EN has been reported to occur in 31%-46% of the patients with NG feeding, some prokinetic agents such as metoclopramide and erythromycin were used to enhance gastric motility and tolerance of enteral feeding. Whether it should be reserved for those patients who are at high risk of upper gastrointestinal intolerance or have already experienced it while receiving NG feeding, requires further studies. Moreover, the optimal dose remains unknown. NJ feeding leads to fewer gastrointestinal complications, largely by reducing gastric residual volumes. So placement of a NJ feeding tube to provide energy support or medication, is increasingly used as a standard clinical practice for many patients[19-21].

But how to place the NJ feeding tube quickly and safely remains an important technique for doctors. The approaches of placing NJ tubes include placement at

surgery, under fluoroscopic or ultrasound-guidance, at endoscopy and blind introduction at the bedside with or without prokinetic administration. The Cathlocator™ is a novel device that permits real time localization of the end of feeding tubes through generating a low energy electromagnetic field from a coil incorporated in the tip of a modified enteral feeding tube connected by wires to a proximal interface. Previously, we would use fluoroscopic placement and/or under direct endoscopic visualization to place NJ tubes. Recently, we have used an ultrathin transnasal endoscope that afforded us a greater success and a shortened procedure time. We evaluated three common methods used to place NJ feeding tubes.

Many studies reported that fluoroscopic guidance in the placement of NJ feeding tubes had a success rate of > 84%[22-24], and endoscopic placement presented a success rate ranging from 90% to 100%[25,26], which are consistent with our outcome. However, fluoroscopic placement exposed patients and doctors to varying doses of radiation. Endoscopic placement procedures are often time consuming, technically cumbersome, and require a significant learning curve[27]. As a result, most gastroenterologists and surgical endoscopists are not satisfied with the current techniques of endoscopic placement. We therefore described a new method to place NJ tubes through guide wire assistance with ultraslim gastroscopy.

Our experience with 29 consecutive guide wire placements of feeding tubes showed that the technique was successful in most patients. Before the operation, we asked the patient whether he/she had received nasal surgery before, and whether accompanied by associated diseases, such as severe bending septum, nasal polyps, severe rhinitis, often epistaxis and other diseases. Six patients who had the aforementioned diseases and subsequently changed to transoral insertion also had the tube placed smoothly in the correct position. In 6 patients with the above complaints, where the assembly was inserted using the transoral route, this did not impair smooth passage of the feeding tube into the correct position. No complications were reported from these methods. Transnasal insertion possessed many advantages compared with other methods. Firstly, the total success rate in the feeding tube placement was high, up to 100%. The operative point was the retropulsion of the feeding tube from the small intestine to the stomach when the endoscope or guide wire was withdrawn. It is not easy to place the guide wire at or beyond the Treitz's ligament using a common endoscope. However, with the transnasal ultraslim endoscope, it became less difficult. Moreover, before inserting the feeding tube along the guide wire, it is very important to lubricate the inner lumen of the feeding tube with paraffin in advance. It not only makes the procedure of withdrawing the guide wire easier, but also avoids pulling out the feeding tube. Secondly, the procedure required less time. In 23 cases with successful one-time transnasal tube placement, the average time required from endoscopic transnasal insertion to the complete removal of the guide wire was only 11.1 ± 2.2 min. Thirdly, the procedure was safe and produced few complications.

In conclusion, our experience showed that the technique of placing NJ feeding tubes with the transnasal ultrathin endoscope is quick, effective and safe.

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COMMENTS

Background

Enteral nutrition (EN) not only provides energy support as with parenteral nutrition, but also maintains the functional intestinal barrier, significantly reducing the incidence of infection and organ failure, shortening hospital stays, and lowering treatment costs. Placement of a nasojejunal (NJ) feeding tube with the aim of providing metabolic support or medication, is increasingly used as a standard clinical practice for many patients.

Research frontiers

NJ feeding tubes positioned beyond the ligament of Treitz's have been shown to allow early attainment of caloric needs and a reduction in tube-feeding aspiration events in patients with gastric feeding intolerance. There are presently several methods for placement of NJ feeding tubes.

Innovations and breakthroughs

The authors used guide wire assistance to place NJ tubes, using an ultrathin transnasal endoscope that afforded them a higher success rate and a shortened procedure time. The authors evaluated the usefulness and safety of the new method compared with the other two traditional methods.

Applications

The technique of placing NJ feeding tubes with the transnasal ultrathin endoscope is quick, effective and safe, which can be applied in clinical practice.

Terminology

Guide wire technology means through guide wire to place NJ feeding tubes. Guide wire was placed beyond the ligament of Treitz's under the ultraslim transnasal endoscope.

Peer review

This article directly compared guide wire method with other two old methods in the duration, success and complications, providing sufficient evidence to prove the superiority of the guide wire method.

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CASE REPORT

Origin of celiac disease: How old are predisposing haplotypes?

Giovanni Gasbarrini, Olga Rickards, Cristina Martínez-Labarga, Elsa Pacciani, Filiberto Chilleri, Lucrezia Laterza, Giuseppe Marangi, Franco Scaldaferri, Antonio Gasbarrini

Giovanni Gasbarrini, Ricerca in Medicina Foundation NGO, Falcone and Borsellino Gallery, 40123 Bologna, Italy
Olga Rickards, Cristina Martínez-Labarga, Center of Molecular Anthropology for Ancient DNA Studies, Department of Biology, University of Rome Tor Vergata, 00173 Rome, Italy
Elsa Pacciani, Filiberto Chilleri, Superintendence for the Archaeological Heritage of Tuscany, 50018 Florence, Italy
Lucrezia Laterza, Franco Scaldaferri, Antonio Gasbarrini, Division of Gastroenterology, Department Internal Medicine, Catholic University of Sacred Heart, 00168 Rome, Italy
Giuseppe Marangi, Institute of Genetics, Catholic University of Sacred Heart, 00168 Rome, Italy
Author contributions: Gasbarrini G and Gasbarrini A designed the research; Rickards O, Martínez-Labarga C designed the molecular research strategy; Rickards O, Martínez-Labarga C, Pacciani E and Chilleri F performed the research; Marangi G analyzed the data; and Rickards O, Martínez-Labarga C, Scaldaferri F and Laterza L wrote the paper.
Correspondence to: Antonio Gasbarrini, MD, Professor of Internal Medicine, Division of Gastroenterology, Department Internal Medicine, Catholic University of Sacred Heart, Largo Gemelli 8, 00168 Rome, Italy. agasbarrini@rm.unicatt.it
Telephone: +39-6-30156265 Fax: +39-6-30155923
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Abstract

We recently presented the case of a first century AD young woman, found in the archaeological site of Cosa, showing clinical signs of malnutrition, such as short height, osteoporosis, dental enamel hypoplasia and cribra orbitalia, indirect sign of anemia, all strongly suggestive for celiac disease (CD). However, whether these findings were actually associated to CD was not shown based on genetic parameters. To investigate her human leukocyte antigen (HLA) class Ⅱ polymorphism, we extracted DNA from a bone sample and a tooth and genotyped HLA using three HLA-tagging single nucleotide polymorphisms for DQ8, DQ2.2 and DQ2.5, specifically associated to CD. She displayed HLA DQ 2.5, the haplotype associated to the highest risk of CD. This is the first report showing the presence of a HLA haplotype compatible for CD in archaeological specimens.

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Key words: Celiac disease; Human leukocyte antigen haplotype; Ancient DNA; Single nucleotide polymorphisms; Malabsorption

Peer reviewers: Dr. Weekitt Kittisupamongkol, Department of Medicine, Hua Chiew Hospital, 665 Bumrungmuang Road, Bangkok 10100, Thailand; Dr. Khaled Ali Jadallah, Department of Internal Medicine, King Abdullah University Hospital, Irbid 22110, Jordan; Dr. Ross McManus, Institute of Molecular Medicine, Trinity Centre for Health Science, St. James's Hospital, Dublin 8, Ireland

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INTRODUCTION

In 2008, we were involved in the "case of Cosa", a skeleton of a young woman dating to the first century AD, found in the archaeological site of Cosa, southwest of Tuscany, Italy (Figure 1)[1]. Based on the physical anthropology description[2], she was a 18-20 year -old woman, dead in physical impairment, showing signs of failure to thrive and malnutrition, all signs of typical celiac disease (CD), in particular, she was slightly built and moderately short for her age (140 cm in height), with clear signs of bone fragility and osteoporosis. She showed on her orbital roof a pathological sign, the "cribra orbitalia" (yet published)[1], a bone porosity also well distinguished in the bone of the skull vault. This condition is generally linked to bone marrow hypertrophy following anemic conditions, such as iron deficient chronic anemia. This

excessive porosity could be also found in the external surface of the bones, in particular in the skull vault (Figure 2A). Furthermore she showed the bone marrow reactive hypertrophy associated to bone atrophy (Figure 2B). Although teeth structure and number were normal, she presented basal dental enamel hypoplasia (Figure 2C), a marker of nutritional or infectious stress. Measuring in the femur the angle between the neck and the diaphysis, it appears larger than normal adult angle (135° *vs* 125°), consistent with a diagnosis of coxa valga, typical of the hip subluxation, due to congenital dysplasia. This diagnosis is supported by the flattening of the postero-superior part of the acetabular cavity. All these signs, taken together, strongly suggested an advanced state of chronic malnutrition, consistent with a typical form of CD[3-5]. From the beginning, a poor availability of food was excluded as several signs indicated that she was member of a wealthy family, as suggested by the jewels she wore and by the overall quality of her tomb. Based on ethologic data[6], we considered that her diet was variegated and probably rich in wheat, and consequently in gluten. We hypothesized that the young woman suffered from CD. Moreover, her death was caused by severe malnutrition or by a complication of it.

The most ancient case of CD ever described was reported by Areteus of Cappadocia in the 1st-2nd centuries AD[7], indicating that CD could have a old origin. However the appearance of the predisposing haplotypes in humans as well as the origin of CD is still unknown.

Genetic susceptibility is a crucial step in the pathogenesis of CD, as demonstrated by studies on monozygotic twins, that show disease concordance rate of 75% compared with 11% in dyzigotic twins[8]. Furthermore siblings have an increased risk of CD, of which about 40% depends on human leukocyte antigen (HLA) genes[9]. The role of HLA class Ⅱ molecules as the major genetic risk factor for CD is well known, even if the genetic effect attributable to HLA is only 54%[10]. It has been reported that over 90% of CD patients express HLA-DQ2 heterodimer while the others express HLA-DQ8[11]. In Europe only fewer than 0.5% of celiac patients express neither DQ2 nor DQ8[12], making HLA genotyping a marker with a very powerful negative predictive value, excluding the diagnosis in people who do not have CD risk HLA haplotype. Until present no information were available on CD genetics in ancient time, since DNA analysis of human remains is technically difficult.

CASE REPORT

In order to confirm the clinical hypothesis that the young woman was actually suffering from CD, we extracted DNA from parts of the skeleton and studied HLA polymorphisms known to be involved in susceptibility to CD. In particular, we analyzed DQ2 (encoded by the *DQA1* 05* and *DQB1*02* alleles) and DQ8 (encoded by the *DQA1*03* and *DQB1*0302* alleles) HLA heterodimers[13,14].

Ancient DNA analysis of human remains is particularly challenging, therefore every attempt was made to ensure the generation of authentic and meaningful data following the strictest available criteria[15-19]. To extract the genetic material, a bone sample and the left mandibular third molar (Figure 2D) were chosen since they are the best sources of DNA. Bones and teeth consist of hard material that contains small hollow spaces with single cells that are less affected by diagenetic processes and by natural contamination (microorganisms, fungus), and modern contaminations are likely to be removed prior to extraction. Bone and tooth sample were firstly brushed and irradiated for 1 h under ultraviolet (UV) light. Afterwards, the entire surface was removed by using dental drills, and the samples were cut into smaller pieces with drill. The samples were again UV-irradiated for 45 min, grounded to fine powder and stored until use at 4 ℃. Only commercially certified DNA/RNA-free consumables were utilized and all tools and containers used were sterile and DNA-free. All workers wear gloves, safety masks, disposable coveralls, plus particular shoes. Every item entering is extensively washed with bleach and subsequently UV-irradiated.

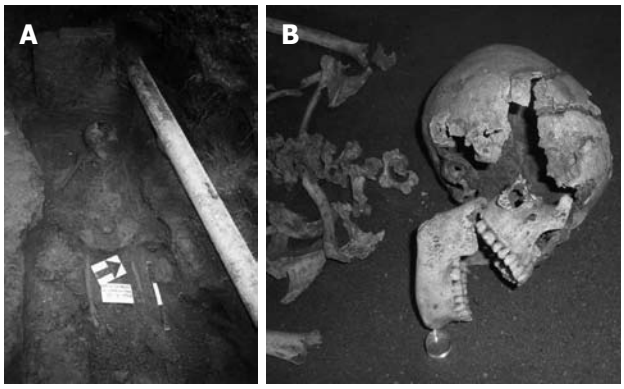


Figure 1 A particular of the skeleton of Cosa in the original site. A: The original site: The ancient remains of Cosa in the original site where they were found; B: The young girl: A particular of the skeleton of the young girl of Cosa.

As first step, we studied the preservation of bone collagen, one of the best indicator of bone preservation and therefore of DNA survival[16]. Collagen was extracted from a small bone fragment following the procedure reported in Craig *et al*[20]. The results obtained indicated the good state of preservation of the collagen. In fact, collagen yield expressed as weight percentage was 1.065% and the ratio C/N was in the range between 3.25 and 3.34 as expected when organic substances are saved from decay[21]. Based on these results, DNA extraction was performed in a laboratory physically isolated from all other laboratories which offers all the state-of-the-art facilities for aDNA studies[17,22,23]. They consist of a contamination resistant facility, which are maintained at positive pressure, frequently cleaned with HCl, NaClO and DNAzap™, UV and high-efficiency particulate arresting filtered, and have restricted access, designed to minimize the possibility of contamination with extant human DNA[23]. The laboratory has consecutive rooms, every room is fitted with UV-C light sources (254 nm) that can be switched on and off from outside the re-



Figure 2 Research on the skeleton of the young girl. A: Skull vault showing excessive porosity; B: A particular of the femur showing cortical atrophy and spongiosum bone hypertrophy, indirect sign of bone marrow hypertrophy, well preserved in 2000 years; C: A detail of teeth, showing basal dental enamel hypoplasia; D: DNA extraction: A tooth and a sample of bone from which DNA was extracted.

Table 1 Primers and length of polymerase chain reaction fragments analyzed for predicted single nucleotide polymorphism

SNPs	Forward primer	Reverse primer	Length fragment (bp)
rs7454108	ACTATTATTTCTCCAAGTTCTGACTTCCCT	GCCAAGTTGGAATAAGCCCACTATA	155
rs7775228	AGGAAAGGAACTATCTGGGTATGGA	TGCAAAGCCCCTTTATCATTATCCT	80
rs2187668	GTGAGGTGACACATATGAGGCAG	GGCTGAATGCCTTCAACAATCATTT	74

SNPs: Single nucleotide polymorphism.

spective lab. The first room has an entry area for changing into suitable clean room clothing. The second room has bench space for handling sampling with fine scale for weighing of samples, a dentist drill for cutting and drilling samples as well as mortars and pestles for griding samples. Another two independent labs with hoods (with internal UV-C sources and biosafety cabinets) are used for DNA extraction and one other for polymerase chain reaction (PCR) setup.

Briefly five hundred milligrams of powder were digested in a proteinase K lysis buffer and DNA was extracted through silica-based spin columns[24]. At least two independent DNA extractions were performed on bone and teeth respectively; mock-extraction controls were carried out identically to those on the samples.

Three HLA-tagging single nucleotide polymorphism (SNPs) were genotyped in order to capture the DQ8, DQ2.2 and DQ2.5 HLA types as reported by Monsuur *et al*[25], using TaqMan chemistry and the on demand assays by Applied Biosystems (Foster City, CA, United States, www.appliedbiosystems.com: Assay IDC_29817179_10dbSNP ID rs7454108; Assay IDC_29315313_10dbSNP ID rs7775228; and Assay IDC_ 58662585_10dbSNP ID rs2187668). Negative controls for amplification (PCR without template DNA) were set up simultaneously to detect contamination and at least 4 independent amplifications of each fragment were performed.

TaqMan® single nucleotide polymorphism (SNP) genotyping assays provide optimized assays for genotyping SNPs and make it easy to perform SNP genotyping discrimination studies. Samples were amplified and genotyped using the manufacturer's instructions on an ABI Prism 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, United States). All SNPs were typed using the standard amplification protocol as supplied by Applied Biosystems (hold 10 min, at 95 ℃, and 40 PCR cycles with denature 15 s, at 92 ℃ and anneal/extend 1 min, at 60 ℃).

Moreover, in order to confirm the RT-PCR results, we amplified and sequenced the three predictive fragments.

The list of primers designed for the experiment and the length of each PCR fragment analyzed are reported in Table 1. PCR amplification was performed in 25 μL reaction containing 2 μL DNA extract, with a final concentration of 1XPCR Gold Buffer Ⅱ, 2.5 mmol $MgCl_2$, 1 mmol dNTPs, 100 nmol primers, 0.1 mg/mL bovine serum albumin, 1 U AmpliTaq Gold (Applied Biosystems). The PCR reaction was run for 35 cycles at 94° for 30 s, 60 ℃ for 30 s and 72 ℃ for 30 s, with a first denaturation step (94 ℃ for 5 min), and a final extension (72 ℃ for 10 min).

PCR products were visualized by gel electrophoresis on a 1.5% agarose gel stained with GelStar (Cambrex, Rockland, ME, United States). Positive amplification products were purified through ExoSap-IT (USB Affymetrix, Santa Clara, CA) according to manufacturer's specifications. Afterwards, they were labeled with fluorescent dyes, purified by the ethanol precipitation technique and submitted to sequencing reaction in an ABI Prism 3100 Avant (Applied Biosystems, Foster City, CA) following the recommended sequencing kit protocols. Sequences were verified through complete overlapping of forward and reverse strands.

Genetic results were independently reproduced multiple times and all sequences were confirmed by at least two different amplified products in order to identify possible contamination.

The young girl turned out to be homozygous FAM for rs7454108, homozygous FAM for rs7775228, and homozygous VIC for rs2187668. The result is compatible with DQ2.5 homozygous genotype which is associated with higher risk of CD. This finding supports on molecular basis our hypothesis that the skeleton found in the site of Cosa suffered from CD.

Finally, to verify the endogenous nature of aDNA and track down any possible modern contaminations, molecular sex and mtDNA characterizations were performed.

Sex determination was carried out by amplification of a segment of the X-Y homologous amelogenin gene using the primer system amelogenin A/B as described by Mannucci *et al*[26]. This method is usually applied for typing samples of a very degraded nature, since short X and Y-specific products of 106 and 112 bp, respectively are generated from a single primer pair. The result were resolved by 12% Acrylamide electrophoresis. Molecular data confirmed the morphological and morphometric sex diagnosis of being female.

Mitochondrial DNA (mtDNA) typing[27] was performed also on the DNAs of all molecular anthropologists and archaeologists who handled the ancient sample. All the extant sequences differed from the girl "Cosa" consensus mtDNA sequence (16270T, 16362C, 73G, 150T and 263G) excluding modern DNA contamination. The ancient haplotype was certainly phylogenetically assigned to U5b2b1a haplo-group following the classification proposed by van Oven *et al*[28]. This haplo-group is European specific and its PAML (Phylogenetic Analysis by Maximum Likelihood) based age estimate is 9325.2 ± 3443.5 years[29-31].

DISCUSSION

This is the first report of HLA typing in ancient remains and it could be considered a very intriguing result, although it does not allow us to diagnose definitively CD. The presence of CD associated-HLA is a necessary condition, although not sufficient to develop the disease. It fact, although about 30%-35% of the actual general population express CD associated HLA genotypes, it has been estimated that, only 2%-5% of risk gene carriers develop the disease[32]. The risk increases further in homozygous for DQ2.5 (*HLA-DQA1*05- DQB1*02*) as shown by a recent study in United States population[12] and by another study exploring relative risks for CD in European population[33]. Another study, on sibs and parents of Italian celiac children, shows that a DQ 2.5 homozigous sib had a risk of 28% of developing CD[34]. Recent advances indicate that other genetic factors may play a role in determining which HLA compatible people could develop CD[35], particularly genes involved in T-cell regulation and inflammation, but they have not been considered for this paper. It has been reported that these genes are contributing for 3%-4% in the risk of CD, together with environmental factors, like early introduction of gluten in infants diet, early infection with enteropathic viruses or the presence of a changed bacterial flora[32].

In the case of "Cosa", even without precise understanding of how environmental factors impacted the girl's life, HLA typing provide us a precious information. The presence of HLA-DQ2.5, in combination with the phenotypic observations, increases the likelihood that the young girl of Cosa suffered from CD and that CD existed already 2000 years ago, like Areteus hypothesized on clinical bases. Our results, strengthen the idea that CD was born a long time ago, walking along together with humans for a long stretch of their history, perhaps even since wheat feeding was introduced.

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CASE REPORT

Biliary tract schwannoma: A rare cause of obstructive jaundice in a young patient

Gilton Marques Fonseca, André Luis Montagnini, Manoel de Souza Rocha, Rosely Antunes Patzina, Mário Vinícius Angelete Alvarez Bernardes, Ivan Cecconello, José Jukemura

Gilton Marques Fonseca, André Luis Montagnini, Manoel de Souza Rocha, Rosely Antunes Patzina, Mário Vinícius Angelete Alvarez Bernardes, Ivan Cecconello, José Jukemura, Digestive Surgery Division, Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, SP 05403-900, Brazil
Author contributions: Fonseca GM and Jukemura J designed research; Fonseca GM and Bernardes MVAA performed research; Montagnini AL, Rocha MS, Patzina RA, Cecconello I and Jukemura J analyzed and reviewed the paper; and Fonseca GM and Jukemura J wrote the paper.
Correspondence to: Gilton Marques Fonseca, MD, Digestive Surgery Division, Department of Gastroenterology, School of Medicine, University of São Paulo, Avenida Doutor Enéas de Carvalho Aguiar, Avenida de Carvalho Aguiar Enéas Doutor, 255 Central Institute, Room 9074, São Paulo, SP 05403-900, Brazil. medgilton@yahoo.com.br
Telephone: +55-11-26617560 Fax: +55-11-26617560
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Abstract

Schwannoma is a tumor derived from Schwann cells which usually arises in the upper extremities, trunk, head and neck, retroperitoneum, mediastinum, pelvis, and peritoneum. However, it can arise in the gastrointestinal tract, including biliary tract. We present a 24-year-old male patient with obstructive jaundice, whose investigation with computed tomography abdomen showed focal wall thickening in the common hepatic duct, difficult to differentiate with hilar adenocarcinoma. He was diagnosed intraoperatively schwannoma of common bile duct and treated with local resection. The patient recovered well without signs of recurrence of the lesion after 12 mo. We also reviewed the common bile duct schwannoma related in the literature and evaluated the difficulty in pre and intraoperative differential diagnosis with adenocarcinoma hilar. Resection is the treatment of choice for such cases and the tumor did not recur in any of the resected cases.

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Key words: Schwannoma; Neurilemoma; Biliary tract; Hilar adenocarcinoma; Computadorized tomography

Peer reviewers: Benito Velayos, PhD, Department of Gastroenterology, Hospital Clínico de Valladolid, Av Ramón y Cajal 3, 47003 Valladolid, Spain; Dr. Hyoung-Chul Oh, Assistant Professor, Department of Internal Medicine, College of Medicine, Chung-Ang University, No. 65, 207 Hanganro-3ga, Yongsan-gu, Seoul 140757, South Korea

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INTRODUCTION

Although it is considered to be a rare tumor, adenocarcinomas are the most common malignant neoplasms of extrahepatic bile ducts[1,2]. However, other non-epithelial tumors can develop, whether they are malignant, such as lymphomas and neuroendocrine cancer[3], or benign, such as adenomas, lipomas, fibromas and schwannomas[4,5], which sometimes appear similar to hilar adenocarcinomas (cholangiocarcinomas).

The goal of this case study was to present a rare case of common bile duct schwannoma that simulated a hilar adenocarcinoma. It was diagnosed during surgery, which allowed only a local resection.

CASE REPORT

A 24-year-old male patient was clinically diagnosed with

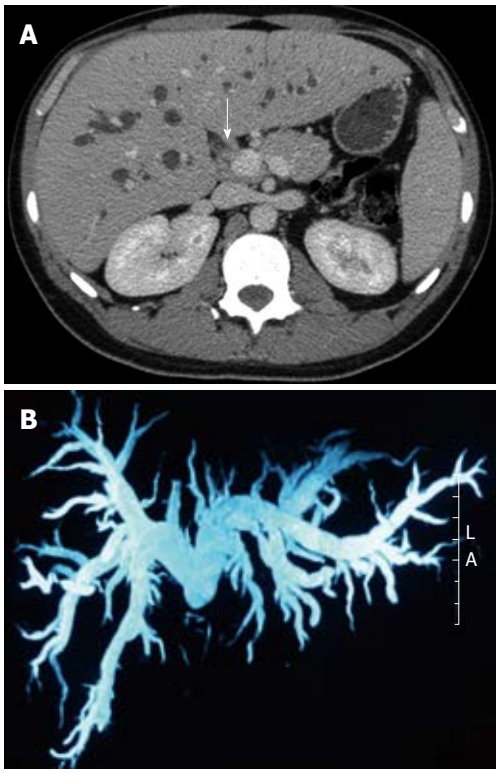


Figure 1 Large dilatation close to hepatic duct. A: Stenosis and thickening of the common hepatic duct (arrow) with upstream biliary dilation; B: Magnetic resonance cholangiography showing large dilatation close to the lesion in the common hepatic duct. A: Ahead; L: Left.

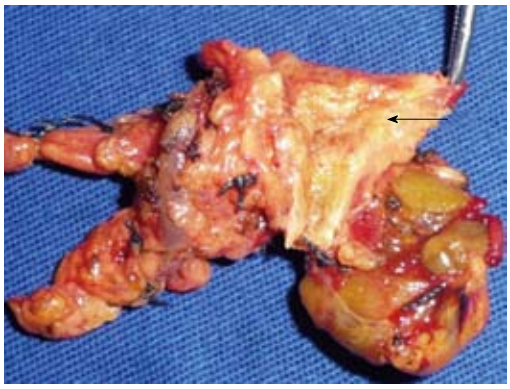


Figure 2 Hepatocholedochal tumor opened longitudinally with the affected area (arrow).

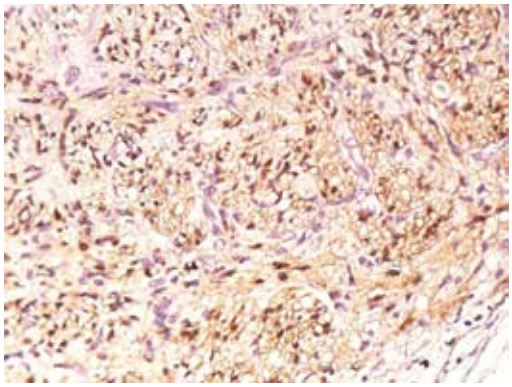


Figure 3 Immunohistochemical staining for the S-100 protein (40 ×).

obstructive jaundice, had 2 mo of epigastric pain and vomiting, and lost 15% of his body weight. The patient had a history of smoking and social drinking. A physical examination confirmed that the patient had jaundice with a flaccid, painless abdomen and palpable liver 4 cm below the right costal margin. There were no other relevant findings.

The laboratory studies revealed: total bilirubin 23.8 mg/dL; direct bilirubin 22.9; serum alkaline phosphatase 298 IU/L; and serum gamma-glutamyl transpeptidase 1052 IU/L; serum asparate aminotransferase 121 IU/L; serum alanine aminotransferase 249 IU/L; and carbohydrate antigen 19-9 was above normal range, 62.7 U/mL. Others laboratory tests were normal.

Imaging studies began with abdominal ultrasound sonography test, which revealed increased liver volume and dilation of the intra-and extrahepatic bile ducts up to the hepatic hilum.

A computed tomography scan of the abdomen showed focal wall thickening in the common hepatic duct 1.5 cm from the hepatic duct confluence. As a result, the diameter of the lumen was reduced, and there was upstream dilation (Figure 1A). The vascular structures and adjacent fat planes were preserved.

A magnetic resonance cholangiography showed biliary tract dilation with abrupt obstruction in the common hepatic duct (Figure 1B).

We opted for surgical treatment without biliary intervention for decompression or endobiliary biopsy, with a preoperative diagnosis of hilar adenocarcinoma. In the cavity, we discovered that the liver was enlarged and appeared to be cholestatic. There was a 1-cm nodular lesion that was palpable in the common hepatic duct; additionally, the proximal bile duct was dilated. A cholecystectomy dissection was performed from the common bile duct above the pancreas to the nodular lesion in the common hepatic duct. During the dissection of the affected area, we observed that the tumor was well defined and regular, with no signs of having infiltrated adjacent tissues (Figure 2). We performed frozen sections, which showed a mesenchymal tumor with free margins in the hepatic duct. In view of these findings, we opted for local resection and Roux-en-Y hepaticojejunostomy.

The patient recovered and was discharged in good condition on the 7th postoperative day. A pathological examination showed a 0.8-cm lesion in the common hepatic duct, which was diagnosed as benign schwannoma with free margins in the extrahepatic bile duct. The diagnosis was made through microscopic examination and immunohistochemistry (Figure 3). The patient was reexamined 12 mo after surgery and was in excellent general health with no signs of recurrence.

DISCUSSION

Benign schwannoma, which is also known as neurile-

Table 1 Cases studies of biliary schwannoma in the literature

Observations/references	Age (yr)	Sex	Signals/ symptoms	Initial diagnosis	Location of tumor	Preoperative tissue acquisition
Oden *et al*[17]	40	F	Abdominal pain + obstructive jaundice	Choledocholithiasis	Commom bile duct	No
Whisnant *et al*[18]	15	F	Abdominal pain + weight loss + obstructive jaundice		Distal portion of the commom bil duct	No
Complicated by liver abscess, treated with drainage/Balart *et al*[19]	56	F	Abdominal pain + obstructive jaundice	Cholangiocarcinoma or extrinsic compression of the bile duct	Commom hepatic duct	No
Jakobs *et al*[20]	37	M	Abdominal pain + obstructive jaundice	Intra-ductal benign tumor	Commom hepatic duct	Yes
Honjo *et al*[13]	48	F	Obstructive jaundice	Benign non-epithelial tumor	Commom bile duct	Yes (transpapillary brush citology, non-diagnostic)
Otani *et al*[21]	59	F	Abdominal pain		Remnant bile duct (pancreatic portion)	No
Park *et al*[22]	53	F	Asymptomatic		Porta hepatis	No
Vyas *et al*[23]	29	F	Abdominal pain + obstructive jaundice		Commom bile duct	Yes (non-diagnostic)
Kamani *et al*[24]	39	F	Jaundice + weight loss	Klatskin tumor	Proximal portion of the commom hepatic duct	No
Fenoglio *et al*[16]	41	F	Obstructive jaundice + weight loss		Middle segment of the commom bile duct	No
Jung *et al*[4]	64	F	Asymptomatic		Proximal portion of the commom bile duct	No
Madhusudhan *et al*[5]	46	M	Obstructive jaundice	Variable polypoid cholangiocarcinoma	Intrahepatic bile duct	Yes
Kulkarni *et al*[7]	38	M	Abdominal pain + weight loss + jaundice		Commom bile duct/ porta hepatis	No
Patient has von Recklinghausen's disease/De Sena *et al*[25]	58	F	Obstructive jaundice	Biliary schwannoma	Extrahepatic bile duct	No
Previous malignant melanoma/Panait *et al*[26]	54	F	Gastroesophageal reflux symptoms	Recurrent metastatic melanoma	Porta hepatis	Yes (non-diagnostic)

F: Female; M: Male.

moma, is a tumor derived from Schwann cells, which form the inner portion of the peripheral nerve sheath[6]. Theoretically, the tumor can affect any organ or nerve trunk, except the optic and olfactory nerves, which lack Schwann cells[7]. The most common locations are the upper extremities, trunk, head and neck, retroperitoneum, mediastinum, pelvis, and peritoneum[8].

Schwannomas in peripheral nerves can be associated with neurofibromatosis type 2, while many schwannomas can occasionally be associated with neurofibromatosis type 1[5,9].

Schwannomas in the digestive tract are relatively rare. These tumors are most common in the stomach followed by the colon/rectum and esophagus[10-12]. Additionally, schwannomas can develop in the biliary tract because there is an abundant network of sympathetic and parasympathetic nerve fibers along the wall of the gallbladder and bile ducts, but these cases are extremely rare[4].

Diagnostic imaging tests do not allow preoperative diagnoses of biliary schwannoma because the findings are similar to those observed in most common lesions, particularly central cholangiocarcinoma. The most relevant finding in our case was that the preoperative examinations revealed a well-defined ductal injury with no signs of adjacent structural involvement or distant metastases[5].

A schwannoma is usually a macroscopically encapsulated solid globular or ovoid tumor. Degenerative cystic changes are occasionally observed within the tumor. Microscopically, the tumor has two components: a hypercellular component with areas of spindle cells forming palisades (Antoni type A) and a myxoid component containing cuboidal cells with clear cytoplasms (Antoni type B)[13].

We found 15 case studies of extrahepatic biliary schwannomas in the literature, which are shown in Table 1. Of these cases, the ages of the patients ranged from 15 to 64 years (with an average age of 44 years), and the patients were predominantly female (12/15). The most common symptom was jaundice (11/15 patients) followed by abdominal pain (7/15) and weight loss (4/15); two patients were asymptomatic. A preoperative diagnosis of hilar adenocarcinoma occurred in 3/15 patients. The lesion was resected in all but one case in which resection was not conducted because of the extensive involvement of the tumor[5]. In this case, endoscopic prosthesis was suggested, but the patient refused the procedure with clinical follow-up. The tumor did not recur in any of the resection cases.

Immunohistochemical analysis is necessary to distinguish schwannomas from neurofibromas, gastrointestinal stromal tumors and leiomyomas. Schwannomas are

strongly positive for vimentin and S100 protein and are negative for muscle cell markers and CD117 (kit), which are found in smooth muscle and gastrointestinal stromal tumors. The CD34 antigen is expressed by a distinct cell population in peripheral nerves, nerve sheath tumors, and related lesions. This antigen is also a useful parameter for the immunohistochemical diagnosis of gastrointestinal stromal tumors. Schwannomas in the digestive tract are usually negative for CD34, although Hou *et al*[14] identified 3 gastrointestinal schwannomas with CD34-positive spindle cells in 33 analyzed cases. Our patient was positive for both the S100 protein, as was expected, and CD34, as in the 3 cases described by Hou *et al*[14].

While there may be preoperative suspicion, the diagnosis of schwannoma requires intraoperative and histopathological confirmation[15]. Despite the possible complications, resection is the treatment of choice for such cases[16]. Schwannomas in the digestive tract have an excellent prognosis after surgical resection, as do schwannomas in other locations. To date, there is no evidence suggesting that these tumors are potentially malignant[4]. In keeping with these findings, our patient remains asymptomatic after 12 mo of postoperative follow-up.

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CASE REPORT

An endoluminal aortic prosthesis infection presenting as pneumoaorta and aortoduodenal fistula

Yung-Ta Kao, Chun-Ming Shih, Feng-Yen Lin, Nai-Wen Tsao, Nen-Chung Chang, Chun-Yao Huang

Yung-Ta Kao, Chun-Ming Shih, Feng-Yen Lin, Nen-Chung Chang, Chung-Yao Huang, Division of Cardiology, Department of Internal Medicine, Taipei Medical University Hospital, Taipei 11031, Taiwan, China
Chun-Ming Shih, Feng-Yen Lin, Nen-Chung Chang, Chun-Yao Huang, Department of Internal Medicine, School of Medicine, College of Medicine, Taipei 11031, Taiwan, China
Feng-Yen Lin, Chun-Yao Huang, Biomedical Apparatus Research Center, Taipei Medical University, Taipei 11031, Taiwan, China
Nai-Wen Tsao, Division of Cardiovascular Surgery, Department of Surgery, Taipei Medical University Hospital, Taipei 11031, Taiwan, China
Author contributions: Kao YT wrote the paper; Shih CM and Lin FY made substantial contributions in reviewing the case and also wrote the paper; Tsao NW described the surgical findings; Chang NC contributed to image compilation; and Huang CY approved the final version of the manuscript.
Correspondence to: Chun-Yao Huang, MD, Division of Cardiology, Department of Internal Medicine, Taipei Medical University Hospital, No. 252 Wu Hsing Street, Taipei 11031, Taiwan, China. cyhuang@tmu.edu.tw
Telephone: +886-2-27372181 Fax: +886-2-27363051
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Abstract

Herein, we present a case of pneumoaorta and aortoduodenal fistula (ADF) caused by an endoluminal aortic prosthesis infection. An 82-year-old man underwent endovascular aneurysm repair with a stent graft to exclude a 5.1-cm abdominal aortic aneurysm. Three months after the index procedure, the patient was taken to the emergency department at a medical university hospital. He presented with a 2-d history of bloody diarrhea. An endoluminal aortic stent graft infection was diagnosed, and an ADF was identified. The patient died of septic shock despite emergency surgery and intensive care. When encountered, stent graft infections require appropriate antibiotics and graft explantation. The diagnosis of an ADF is important, and surgery remains the most effective management if septic shock presents despite conservative treatment.

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Key words: Aortoduodenal fistula; Endovascular aneurysm repair; Infection; Stent graft; Shock

Peer reviewer: Dr. Guideng Li, Department of Biological Chemistry, University of California, 3101 Hewitt Hall, Irvine, CA 92697-4120, United States

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INTRODUCTION

The use of stent grafts to treat abdominal aortic aneurysms (AAAs) has recently become more widely used[1-3]. Stent graft infection rates associated with use of the endovascular technique appear to be lower than those for conventional open repair (range: 0.3%-0.4%)[1,2] as demonstrated by Ducasse *et al*[3], who reported a frequency of infection of 0.43%. However, the incidence of infection resulting from the implantation of an endoluminal aortic prosthesis has been reported to be as high as 6%[4,5]. Although prophylactic antibiotics are routinely prescribed prior to an operation, the incidence of infection and possible sequelae remain difficult to predict. Surgical intervention with complete stent graft removal may provide the best outcome for patients with an infection[3]. The overall treatment strategy can be optimized with the early detection of an endovascular stent graft infection. Herein, we present the case of an elderly male patient with an

endovascular stent graft infection who ultimately died of septic shock despite intensive care.

CASE REPORT

An 82-year-old male patient with type 2 diabetes initially presented with a bulging mass in the abdomen and localized pain. The patient underwent endovascular aneurysm repair with a stent graft (Zenith; Cook, Bloomington, IN) to exclude a 5.1-cm AAA in October 2010 (Figure 1A). The patient recovered without incident post-surgery and was discharged. Although the post-surgical protocol calls for an abdominal computed tomography (CT) scan at one month, the patient did not return to the cardiothoracic surgery clinic following his discharge.

Three months post-surgery, the patient was taken to an emergency department at a medical university hospital reporting a 2-d history of bloody diarrhea. Upon further examination, his blood pressure was measured at 121/55 mmHg, his body temperature was 37.8 ℃, his pulse was 68/min, and his oxygen saturation was 88% as measured using a nasal cannula with 3 L/min of oxygen flow. A physical exam revealed pale conjunctiva and a distended abdomen. Furthermore, blood analysis revealed leukocytosis with a white blood cell count of 21 250 cells/mm^3 and a hemoglobin level of 9.6 g/dL. Serum chemistry was unremarkable except for mildly elevated creatinine (1.3 mg/dL) with an estimated glomerular filtration rate of 56.45 mL/min. Empiric antibiotics were prescribed, including flomoxef and vancomycin. An abdominal CT scan disclosed a fistula between the aorta and the retroperitoneal duodenum, suggesting the formation of an aortoduodenal fistula (ADF) (Figure 1B). There was circumferential fluid collection with air surrounding the stent region, suggesting the presence of necrotic tissue and associated gas formation (Figure 1B-D). Due to the occurrence of refractory shock, an emergency operation was indicated. A bilateral axillary-femoral extra-anatomic bypass was performed with 8-mm polytetrafluoroethylene grafts. In addition, a retroperitoneal abscess and an abdominal aortic aneurysmal sac necrosis were debrided. The ADF was located at the bare-metal stent supra-renal fixation point. The stent graft was removed, and the aortic stump was closed just distal to the renal artery orifices. The tear at the third portion of the duodenum was repaired using LigaSure (Tyco-Healthcare, United States). A segmental resection of the duodenum and a side-to-end duodenojejunostomy were performed. The ADF was 1.5 cm × 1.3 cm in diameter.

Blood cultures revealed a mixed growth of *Salmonella* species, *Bacteroides fragilis*, *Clostridium* species and *Gemella morbillorum*. Bacterial culture of the necrotic tissue demonstrated a mixed Salmonella and Bacteroides infection. The patient died of septic shock two days after admission despite intensive care.

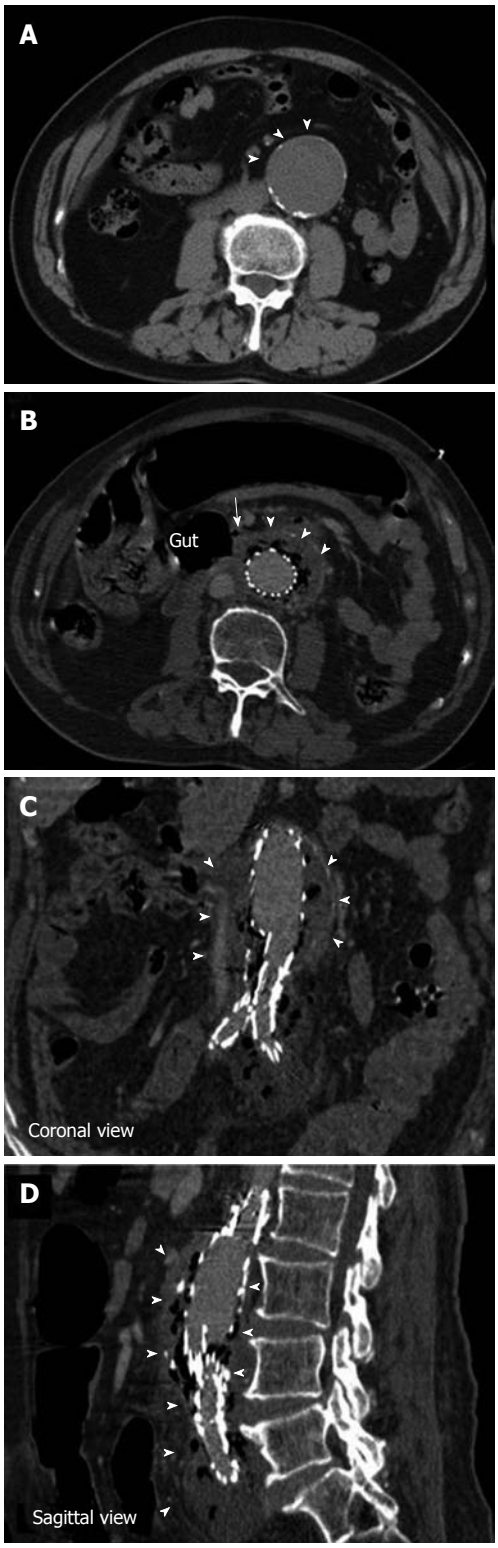


Figure 1 Abdominal computed tomography scans of the patient. A: The abdominal aortic aneurysm before stent-graft implantation (arrowheads); B: The aortoduodenal fistula (arrow); B-D: The necrotic tissue and associated gas formation (arrowheads).

DISCUSSION

In the current case, a gas-forming bacterial infection resulted in the development of a pneumoaorta, which is uncommon. In one recent report[6], a 77-year-old man was diagnosed with a stent graft infection, and his CT scan demonstrated soft-tissue thickening and air present in the right anterolateral aspect of the aneurysm sac. Ad-

ditionally, coagulase-negative staphylococci were identified in a blood culture. Another case report[7] described a stent graft infection due to *Bacteroides fragilis*. The patient's condition was successfully managed with staged extra-anatomic revascularization followed by graft excision.

ADF is a well-recognized and dangerous condition[8-15]. According to a single-center review, five patients developed an ADF between 18 d and 1 year after successful endovascular aneurysm repair[12]. ADF has also been shown to occur as late as five years after endovascular aneurysm repair[13]. The patient in this study presented with ADF and bloody stool passage three months after the index procedure. ADFs and aortic aneurysms can be caused by biliary stent-induced retroperitoneal perforation[14]. However, the current patient did not report any prior gastrointestinal (GI) procedures, such as biliary stenting. The ADF etiology might have included infection and endoleak[12], but an endoleak was not observed on the CT scan. An ADF might further act as a connecting route between the GI tract and the aorta, causing bacterial propagation and infection-related deterioration. Therefore, the bloody stool passage was a possible sequela of the ADF formation[15].

In conclusion, prompt diagnosis and intervention are crucial for effectively treating a patient with an endovascular stent graft infection. A combination of the appropriate antibiotics and surgical repair is the best course for avoiding a fatal outcome. The most effective surgical intervention consists of a complete stent graft explantation followed by *in situ* reconstruction. Endovascular prosthesis implantation is a challenging technique for AAA, but the early recognition and detection of a possible stent infection may be more critical.

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CASE REPORT

Penicillium marneffei chylous ascites in acquired immune deficiency syndrome: A case report

Yin-Zhong Shen, Zhen-Yan Wang, Hong-Zhou Lu

Yin-Zhong Shen, Zhen-Yan Wang, Hong-Zhou Lu, Department of Infectious Diseases, Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, China
Author contributions: Shen YZ, Wang ZY and Lu HZ provided medical care; and Shen YZ wrote the paper.
Correspondence to: Dr. Hong-Zhou Lu, Department of Infectious Diseases, Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, China. luhongzhou@fudan.edu.cn
Telephone: +86-21-57248758 Fax: +86-21-57248758
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Abstract

Penicillium marneffei (*P. marneffei*) infection usually occurs with skin, bone marrow, lung or hepatic involvement. However, no cases of *P. marneffei* infection with chylous ascites have been reported thus far. In this report, we describe the first case of acquired immune deficiency syndrome (AIDS) which has been complicated by a *P. marneffei* infection causing chylous ascites. We describe the details of the case, with an emphasis on treatment regimen. This patient was treated with amphotericin B for 3 mo, while receiving concomitant therapy with an efavirenz-containing antiretroviral regimen, but cultures in ascitic fluid were persistently positive for *P. marneffei*. The infection resolved after treatment with high-dose voriconazole (400 mg every 12 h) for 3 mo. *P. marneffei* should be considered in the differential diagnosis of chylous ascites in human immunodeficiency virus patients. High-dose voriconazole is an effective, well-tolerated and convenient option for the treatment of systemic infections with *P. marneffei* in AIDS patients on an efavirenz-containing antiretroviral regimen.

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Key words: Chylous ascites; *Penicillium marneffei*; Acquired immune deficiency syndrome; Voriconazole; Efavirenz; Fungal sepsis

INTRODUCTION

Penicillium marneffei (*P. marneffei*) infection now represents one of the most common acquired immune deficiency syndrome (AIDS)-defining opportunistic infections in endemic areas of Southeast Asia[1-3]. The infection is associated with a high mortality rate if timely treatment with appropriate antifungal drugs is not administered[2,4]. *P. marneffei* usually occurs with skin, bone marrow, lung or hepatic involvement; however, no cases of *P. marneffei* infection with chylous ascites have yet been reported. In this report, we describe the first case of AIDS which has been complicated by a *P. marneffei* infection causing chylous ascites. We describe the details of the case with emphasis on treatment regimen.

CASE REPORT

A 47-year-old man, a native of Yunnan province, southwest China, infected with human immunodeficiency virus (HIV) who had a CD4 cell count of 66 cells/μL had a 1-year history of intermittent fever and a 6-mo history of abdominal distension. Culture of blood and ascitic fluid

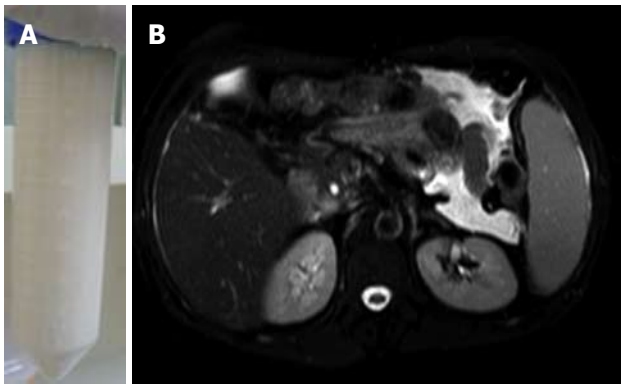


Figure 1 The auxiliary examination for the patient. A: The ascitic fluid had a milky appearance; B: Abdominal magnetic resonance imaging showed hepatosplenomegaly with ascites.

revealed *P. marneffei*. He was diagnosed with AIDS and *P. marneffei* infection. He had received antiretroviral therapy (ART) with stavudine (30 mg twice daily), lamivudine (300 mg once daily) and efavirenz (600 mg daily before bedtime). He was treated with amphotericin B 25 mg/d which was continued for 3 mo, but his condition did not improve. Persistent abdominal distension remained, so paracentesis was performed periodically to relieve the symptoms. Cultures in ascitic fluid were persistently positive for *P. marneffei*. He came to our hospital for further management. Examination revealed the presence of shifting dullness and paracentesis confirmed diagnosis of chylous ascites. Total serum protein, albumin, total cholesterol and triacylglycerol were 65.80 g/L, 37.70 g/L, 3.92 mmol/L and 0.95 mmol/L, respectively. The ascitic fluid had a milky appearance (Figure 1A) and showed 270 white blood cells/μL with 60% multinucleated cells and 40% lymphocytes, triglyceride 13.59 mmol/L (normal range < 1.70 mmol/L), and total protein 31.5 g/L. No malignant cells were found on pathology. Chest computer tomography scan showed no abnormalities while abdominal magnetic resonance imaging showed hepatosplenomegaly with ascites (Figure 1B). Treatment with oral voriconazole (400 mg every 12 h) was started and was continued for 3 mo. He continued on ART (stavudine, lamivudine and efavirenz), but he was recommended to take a lower dose of efavirenz (300 mg once daily before bedtime). In order to monitor efficacy and side effects, we performed therapeutic drug concentration monitoring (TDM) of both voriconazole and efavirenz. The results showed that the peak and trough concentrations of voriconazole were 2.31 mg/L and 1.42 mg/L, respectively and the peak and trough concentrations of efavirenz were 3720 ng/mL and 2680 ng/mL, respectively. While on voriconazole, he improved. All disease-related clinical symptoms and signs gradually disappeared. Fungal cultures of chylous ascites became negative after receiving 2 wk of voriconazole treatment. The values of laboratory tests including electrolytes, renal function and transaminases were within normal ranges during treatment. The infection resolved after treatment with voriconazole for 3 mo and was discontinued when recovery was thought to be achieved. He was recommended to take a normal dose of efavirenz (600 mg once daily) after discontinuation of voriconazole. Six months after initiation of ART, his CD4 cell count rose to 110 cells/μL. He refused to receive secondary prophylaxis with antifungal drugs. No relapse was found in an 8-mo follow-up.

DISCUSSION

Prior to the epidemic of AIDS, penicilliosis was a rare event. The incidence of this fungal infection has increased markedly during the past few years, paralleling the incidence of HIV infection[1]. In China, *P. marneffei* infection is endemic in Guangdong, Guangxi, Yunnan, HongKong and Taiwan[5-7]. In recent years, with the increase of HIV infection, cases have been reported from non-endemic regions[3,8].

Chylous ascites is a rare complication of AIDS. It is caused by the leakage of chyle into the peritoneum due to rupture or obstruction of the thoracic duct. *P. marneffei* is a systemic fungal infection which usually occurs with skin, bone marrow, lung or hepatic involvement. This patient is the first case of AIDS which has been complicated by a *P. marneffei* infection causing chylous ascites. This case suggests that *P. marneffei* should be an important differential diagnosis in chylous ascites in HIV patients, especially in visitors to, or residents of, endemic areas for *P. marneffei*.

The recommended treatment for *P. marneffei* is amphotericin B for 2 wk, followed by oral itraconazole for a subsequent duration of 10 wk[9]. ART should be administered simultaneously to improve outcome[9], but avoiding adverse drug interactions between antifungals and antivirals is also important as these can complicate therapy. This patient had failed to respond to initial amphotericin B treatment. *P. marneffei* is highly susceptible to miconazole, itraconazole, ketoconazole, and 5-flucytosine. Amphotericin B has intermediate antifungal activity[9] which means some patients may not respond to treatment with it. Up to now, alternative treatment options for penicilliosis have not been established. A small case series[10] reported good outcomes with voriconazole in AIDS patients with systemic *P. marneffei* infections, but none of the patients in that study received ART simultaneously. Efavirenz is a mixed inducer and inhibitor of CYP3A4, 2C9 and 2C19. Concomitant use of itraconazole and efavirenz may result in subtherapeutic levels of itraconazole[9], so use of an alternate antifungal treatment may be necessary. Alternatively efavirenz can be replaced with a noninducing class of antiretrovirals. The limited number of antiviral drugs limits the choice of treatment of AIDS in China. We chose voriconazole as the alternative treatment option for penicilliosis. Voriconazole is metabolized primarily by CYP2C19, as well as CYP2C9 and CYP3A4[11]. Voriconazole is also known to inhibit these enzymes[11], and the manufacturer reports an extensive list of drugs that interact with voriconazole. Efavirenz reduces the plasma concentration of voriconazole which increases the plasma concentration of efavirenz,

thus dose adjustments for voriconazole and efavirenz may be needed if concomitant use of these two agents is necessary[9]. It is recommended that clinicians increase voriconazole to 400 mg every 12 h and decrease efavirenz to 300 mg once daily before bedtime[9]. Therefore, we increased the voriconazole dose to 400 mg twice daily and decreased the efavirenz dose to 300 mg once daily before bedtime.

A relationship between progression of fungal infection and voriconazole concentrations was demonstrated in several studies[12-14]. They showed that monitoring voriconazole concentration and adjusting dosage to attain an appropriate plasma concentration is necessary to ensure antifungal effect and to avoid toxicity. TDM represents an important process to optimize the outcome of immunocompromised patients receiving triazoles[15]. The patient was treated with amphotericin B followed by 3 mo of high-dose voriconazole therapy, which resulted in a clinical cure. Treatment with high-dose voriconazole was well tolerated, with no discontinuations caused by drug-related adverse events. The patient also received ART containing low-dose efavirenz, without any relapse of *P. marneffei* infection. Treatment with low-dose efavirenz in this patient was also effective, with clinically significant increases in his CD4 counts. The results of this study suggest that high-dose voriconazole is an effective, well-tolerated, and convenient option for the treatment of systemic infections with *P. marneffei* in AIDS patients on efavirenz-containing ART. There may be a role for voriconazole and efavirenz serum concentration monitoring to ensure therapeutic efficacy when the drugs are used concomitantly.

All patients who successfully complete treatment for penicilliosis should receive oral itraconazole as maintenance therapy to prevent relapse[9]. Discontinuation of secondary prophylaxis is recommended for AIDS patients who receive ART and have CD4 count > 100 cells/μL for > 6 mo [9]. This patient did not receive secondary prophylaxis with antifungal drugs. He had a CD4 count > 100 cells/μL 6 mo after initiation of ART. During the follow-up, no relapse of the fungal infection was observed.

In conclusion, this case report indicates that penicilliosis marneffei should be considered in the differential diagnosis of chylous ascites in HIV patients. High-dose voriconazole is an effective, well-tolerated, and convenient option for the treatment of penicilliosis marneffei in AIDS patients on efavirenz-containing ART. Further research into alternative treatment options for penicilliosis in AIDS patients is required.

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Vague relationship between alcohol consumption and metabolic syndrome in nonobese people

Kei Nakajima, Masafumi Saito

Kei Nakajima, Masafumi Saito, Division of Clinical Nutrition, Department of Medical Dietetics, Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan
Author contributions: Nakajima K and Saito M researched and evaluated the literature; Nakajima K wrote the manuscript; and both authors reviewed and edited the manuscript, and approved the final version of the manuscript.
Correspondence to: Kei Nakajima, MD, PhD, Division of Clinical Nutrition, Department of Medical Dietetics, Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan. keinaka@josai.ac.jp
Telephone: +81-49-2717260 Fax: +81-49-2717260
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Abstract

Fatty liver, including non-alcoholic fatty liver disease, is closely associated with metabolic syndrome (MS). Thus, the presence of fatty liver without MS in some conditions may be clinically important. Many studies have shown that compared with no or occasional alcohol intake, moderate alcohol consumption is associated with lower prevalence rates of hypertension and type 2 diabetes, and lower levels of circulating C-reactive protein, a valuable marker for MS and insulin resistance. Considering these findings, light to moderate alcohol consumption has theoretical benefits on fatty liver and MS. Fatty liver, including non-alcoholic fatty liver disease, may be more clinically important than MS, particularly in non-obese individuals, because fatty liver can develop before MS in several conditions, such as regular alcohol consumers. Furthermore, most of the currently used MS criteria are unable to detect "true MS" because of variations in multiple factors such as age, height, medications, and complications.

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Key words: Alcohol consumption; Non-alcoholic fatty liver disease; Metabolic syndrome; Adult treatment panel Ⅲ

Peer reviewers: Alessandro Grasso, MD, Internal Medicine and Gastroenterology Unit, San Paolo Hospital, 17100 Savona, Italy; Yusuf Yilmaz, MD, Department of Gastroenterology, School of Medicine, Marmara University, Fevzi Cakmak Mah, Mimar Sinan Cad. No. 41 Ust Kaynarca, Pendik, 34899 Istanbul, Turkey; Carlos A Aguilar-Salinas, MD, Department of Endocrinology and Metabolism, Instituto Nacional de Ciencias Medicas y Nutricion, Vasco de Quiroga 15, Mexico City 14000, Mexico

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TO THE EDITOR

We read the recent article by Hamaguchi *et al*[1] with much interest. The authors showed in a cross-sectional study that the effects of alcohol consumption differed between metabolic syndrome (MS) and fatty liver, and that light to moderate alcohol consumption had a favorable effect on fatty liver, but not on MS, in Japanese men and women. Habitual alcohol consumption, which generally impairs fatty acid oxidation and stimulates lipogenesis in the liver[2,3], substantially influences morbidity, all-cause mortality, and cardiovascular mortality rates[4,5]. Thus, the findings reported by Hamaguchi *et al*[1] are impressive in terms of public health and scientific interest. As described by the authors, fatty liver, including non-alcoholic fatty liver disease (NAFLD), is closely associated with MS and is a hepatic manifestation of MS or insulin resistance. This is based on the concept that MS is a leading cause for fatty liver in the cause-effect relationship.

In addition, many studies have shown that moderate alcohol consumption is associated with a low incidence of adverse health outcomes associated with hypertension[5] and type 2 diabetes[6], and with lower levels of cir-

culating C-reactive protein (CRP)[7,8], an important marker for MS, obesity, and insulin resistance[9,10]. The association between moderate alcohol consumption and lower CRP was independent of body mass index (BMI)[9,10] and alcohol-related effects on lipids[10].

Considering these findings, light to moderate alcohol consumption is hypothesized to have beneficial effects on fatty liver and MS. Plausible explanations for the lack of a beneficial effect of light to moderate alcohol consumption on the risk of MS include the characteristics of the subjects studied, the criteria used to define MS, or food patterns specific for light to moderate alcohol consumers. Of particular note, NAFLD also occurs, albeit relatively infrequently, in normal weight people who commonly have metabolic abnormalities and insulin resistance[11,12]. Most of the subjects in the study by Hamaguchi *et al*[1] were of normal weight (mean BMI 23.2-23.5 kg/m^2 for men and 20.9-21.4 kg/m^2 for women), in whom the prevalence of MS is likely to be reduced when waist circumference is dichotomized according to the criteria for MS with a fixed threshold. High waist circumference is necessary for the diagnosis of MS according to the International Diabetes Federation (IDF) criteria for MS, but not the adult treatment panel (ATP)-Ⅲ criteria for MS. This may help to explain the lower prevalence of people fulfilling the IDF criteria than the ATP-Ⅲ criteria in that study. By contrast, fatty liver can be diagnosed using ultrasound techniques irrespective of manufacturer-specific criteria, which likely resulted in the unexpected finding that more than half of the subjects with fatty liver did not have MS.

Furthermore, although the authors claimed in the discussion that there was no significant association between alcohol consumption and BMI > 25.0 kg/m^2, the prevalence of BMI > 25.0 kg/m^2 was actually lower, particularly in females with light to moderate alcohol consumption. This suggests that the observed association might be mediated by lower BMI, which is also affected by dietary patterns and nutrient intake in such alcohol consumers[13,14]. Sub-analyses of subjects divided into specific groups (e.g., overweight/obese groups) or analyses controlling for BMI, waist circumference, and dietary patterns (as assessed by food frequency questionnaires, for example) are needed to clarify whether the observed association is independent of obesity, MS criteria, or dietary patterns.

Taken together, the results of the study by Hamaguchi *et al*[1] provide meaningful insight into the etiological differences in metabolic abnormalities between liver and visceral adipose tissue, in which lipolysis is reduced by acetate produced in the liver[15]. In addition, the results indicate that fatty liver, including NAFLD, may be clinically more important than MS, particularly in non-obese people, because fatty liver can develop before MS in some conditions and that MS criteria are often unable to detect "true MS". The authors propose that fatty liver without MS is an important disease in the general population. Similarly, Stefan *et al*[16] proposed that fatty liver may be more important than visceral fat for the discrimination of benign obesity that is not accompanied by insulin resistance.

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Elke Cario, MD, Professor, Division of Gastroenterology and Hepatology, University Hospital of Essen, Medical School, University of Duisburg-Essen, Institutsgruppe I, Virchowstr. 171, 45147 Essen, Germany

Adrian G Cummins, Associate Professor, Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia

Myung-Gyu Choi, MD, Professor of Medicine, Division of Gastroenterology, Department of Internal Medicine, Seoul St. Mary's Hospital, The Catholic University of Korea, 505, Banpo-Dong, Seocho-Gu, Seoul 137-040, South Korea

Seng-Kee Chuah, MD, Division of Hepatogastroenterology, Chang Kaohsiung Gang Memorial Hospital, 123, Ta-Pei Road, Niaosung Hsiang, Kaohsiung 833, Taiwan, China

Filip Braet, Associate Professor, Australian Key Centre for Microscopy and Microanalysis, Madsen Building F09, The University of Sydney, Sydney, NSW 2006, Australia

De-Liang Fu, MD, PhD, Professor, Department of Surgery, Pancreatic Disease Institute, Fudan University, Shanghai 200040, China

Ching Chung Lin, MD, MMS, Division of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei 111, Taiwan, China

Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, H1083 Budapest, Hungary

Steven Hochwald, MD, Associate Professor, Chief, Department of Surgery, University of Florida, 1600 SW Archer Rd, PO Box 100109, Gainesville, FL 32610, United States

Ibrahim A Al Mofleh, Professor, Deaprtment of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Maha Maher Shehata, Professor, Internal Medicine, Department/Gastroenterology and Hepatology Unit, Medical Specialized Hospital, 35516 Mansoura, Egypt

Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor, Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Sang Min Park, MD, MPH, PhD, Assistant Professor, Department of Family Medicine, Seoul National University Hospital, 101 Daehangno, Jongno-gu, Seoul 110-744, South Korea

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Andrada Seicean, MD, PhD, Third Medical Clinic Cluj Napoca, University of Medicine and Pharmacy Cluj Napoca, Romania, 15, Closca Street, 400039 Cluj-Napoca, Romania

Yoshio Shirai, Associate Professor, Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Niigata City 951-8510, Japan

Naoaki Sakata, MD, PhD, Division of Hepato-Biliary Pancreatic Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

Masayuki Sho, MD, PhD, Professor, Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan

John B Schofield, MB, BS, MRCP, FRCP, Department of Cellular Pathology, Preston Hall, Maidstone, Kent ME20 7NH, United Kingdom

Yoshitaka Takuma, MD, PhD, Department of Gastroenterology, Kurashiki Central Hospital, 1-1-1 Miwa, Kurashiki, Okayama 710-8602 Japan

Wei-Dong Tong, MD, PhD, Associate Professor, Daping Hospital, Third Military Medical University, Chongqing 400042, China

Jiping Wang, MD, PhD, Division of Surgical Oncology, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, United States

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London, United Kingdom

February 16-17, 2012
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Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

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Orlando, FL 32808, United States

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9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

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Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

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The International Liver Congress 2012
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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
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EDITORIAL

Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy: Where are we?

Ingmar Königsrainer, Stefan Beckert

Ingmar Königsrainer, Stefan Beckert, Department of General, Visceral and Transplant Surgery, Tübingen University Hospital, D-72076 Tübingen, Germany
Author contributions: Königsrainer I prepared the first draft and designed the editorial; Beckert S designed the editorial and revised the manuscript; both authors performed the literature search and approved the final manuscript.
Correspondence to: Ingmar Königsrainer, MD, Department of General, Visceral and Transplant Surgery, Tübingen University Hospital, Hoppe-Seyler-str. 3, D-72076 Tübingen, Germany. ingmar.koenigsrainer@med.uni-tuebingen.de
Telephone: +49-7071-2985073 Fax: +49-7071-295588
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Abstract

Peritoneal surface malignancies are generally associated with poor prognosis. In daily clinical routine, systemic chemotherapy is still considered the only reasonable therapy despite of encouraging results of cytoreductive surgery (CRS) along with intraperitoneal hyperthermic chemotherapy (HIPEC). The Achilles heel of CRS and HIPEC is appropriate patient selection and precise surgical technique preventing patients from excessive morbidity and mortality. Given these findings, new concepts of second look surgery for high risk patients allow detection of peritoneal spread ahead of clinical symptoms or presence of peritoneal masses reducing perioperative morbidity. In addition, personalized intraperitoneal chemotherapy might further improve outcome by appreciating individual tumor biology. These days, every physician should be aware of CRS and HIPEC for treatment of peritoneal surface malignancies. Since there is now sufficient data for the superiority of CRS and HIPEC to systemic chemotherapy in selected patients, our next goal should be providing this strategy with minimal morbidity and mortality even in the presence of higher tumor load.

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Key words: Peritoneal carcinomatosis; Hyperthermic intraperitoneal chemotherapy; Cytoreductive surgery

Peer reviewers: Jean-Jacques Tuech, MD, PhD, Department of Digestive Surgery, Rouen University Hospital, 1 rue de Germont, 76031 Rouen, France; Jens Hoeppner, MD, Assistant Professor, Department for General and Visceral Surgery, Universitaetsklinikum Freiburg, Hugstetterstr. 55, 79106 Freiburg, Germany; Patrick O'Dwyer, MB, BCh, BAO, FRCS (1), MCh, FRCS (Glasg), University Department of Surgery, Western Infirmary, Glasgow G11 6NT, United Kingdom

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INTRODUCTION

In Peritoneal surface malignancies (PSM), tumor location is restricted to the abdominal cavity as opposed to systemic metastatic disease. In the past, the majority of PSM patients underwent systemic chemotherapy which was associated with poor quality of life and was ineffective with respect to prolonging survival.

Thanks to the pioneer work of Professor Paul Sugarbaker, a proactive surgical approach termed cytoreductive surgery (CRS), aiming for maximal tumor resection, along with hyperthermic intraperitoneal chemotherapy (HIPEC) evolved into a highly relevant treatment option for selected patients with limited peritoneal spread of various tumor entities[1-3]. The rationale for this particular approach is the restriction of tumor dissemination to the peritoneal compartment justifying a radical surgical procedure followed by HIPEC.

Although there is now evidence for the superiority of

CRS and HIPEC to systemic chemotherapy, this strategy has not made its way into clinical routine since peritoneal spread is still considered as stage Ⅳ cancer when surgical resection is not an option any more. However, there was a similar thinking for colorectal liver metastasis for a long time. Now, surgery represents the main strategy even though its superiority has never been proven in a randomized phase Ⅲ trial.

If cytoreductive surgery is scheduled, proactive surgery achieving total or almost total (remaining nodules < 2.5 mm) cytoreduction has to be the main aim In addition, hyperthermic intraperitoneal chemotherapy is administered for eradication of microscopic residual disease. The most frequently cited paper on this topic was published by Verwaal *et al*[4,5] who first proved the benefit of this multimodal approach in a phase Ⅲ trial comparing patients with colorectal cancer undergoing CRS and HIPEC followed by systemic chemotherapy with systemic chemotherapy. Up to now, there are several reports on long term survival if radical resection was performed[4-7]. Other entities for which this treatment is accepted are pseudomyxoma peritonei and mesothelioma[8]. For selected patients with ovarian and gastric cancer this option can be offered with good results[9-12].

Since survival does not significantly differ between completeness of cytoreduction CC0 or CC1, an oncologic resection with wide resection margins seems not necessary in this content except for primary gastrointestinal cancer with peritoneal carcinomatosis PC.

INTERDISCIPLINARY CONCEPT

The implementation of many new centers for PSM could mean that more and more patients are asking for this therapeutic option. However, the perioperative setting has to be established first rather than the surgical one. An experienced radiologist is mandatory to assess preoperative tumor load and to rule out contraindications such as diffuse infiltration of the small bowel or extraperitoneal disease. The anaesthesiologists, nurses and HIPEC technicians should visit centers and participate in workshops for HIPEC before initiating the program. Lastly, the medical oncologist becomes more and more important because there are numerous different intraperitoneal as well as pre- and postoperative chemotherapy regimens. The founding of a peritoneal surface malignancy group which meets regularly has had a great impact on scientific discussion between surgeons, radiologists, anaesthesiologists. In addition, this facilitates the initiation of clinical multi-center and experimental studies.

HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY

In most cases, intraperitoneal chemotherapy is administered after cytoreductive surgery and completion of intestinal anastomoses, either immediately intraoperatively (HIPEC) or early postoperatively (EPIC). Technically, this chemotherapy can be applied to an open or closed abdomen which varies between the different centers.

The rationale of HIPEC is the synergistic cytotoxic effect of heat, ideally 42-43 degree Celcius, and the chemotherapeutic agent itself on tumor cells.

There are various concepts varying in duration of exposition, in combination with for example intraoperative intravenous therapy and in type of the administered chemotherapy.

The effect of hyperthermic intraperitoneal chemotherapy itself has never been proven in a randomized controlled trial and is still the focus of ongoing investigations. Nevertheless, there are numerous data of how HIPEC might work and most surgeons, medical oncologists and last but not least patients believe in the effect of local chemotherapy.

The rationale for applying intraoperative chemotherapy under hyperthermic conditions is improving both tissue as well as tumor oxygenation by vasodilation enhancing the cytotoxic effect of chemotherapeutic agents. So far, however, nobody has demonstrated an effect on hyperthermia on tissue oxygenation and there is no data whether this putative effect on pO_2 (oxygen) might be sustained throughout the entire HIPEC period. As learned from wound healing research, supplemental oxygen during HIPEC might further enhance cytotoxicity since it has been shown to increase tissue oxygen tension. In addition of thinking about the best timing for HIPEC, HIPEC in combination with supplemental oxygen could be a worthwhile option in the future.

Another future important issue could be testing chemotherapeutic sensitivity to improve the cytotoxic effect of HIPEC. Such particular tests already exist for ovarian cancer with respect to platinum resistance[13,14]. This further strengthens the need for personalized intraoperative chemotherapy regimens.

NEOADJUVANT CHEMOTHERAPY

A quite high percentage of patients is not eligible for cytoreductive surgery at the time of surgical exploration. Therefore, tumor downsizing by systemic chemotherapy and subsequent surgery might be an option. In liver surgery, the concept of secondary resection after chemotherapy, both intravenous as well as regionally, is accepted and response to preoperative chemotherapy can be considered as a prognostic factor[15-17]. In PSM, neoadjuvant chemotherapy might also aid in categorizing patients in responders and non-responders with responders being more likely to profit from CRS and HIPEC. One limitation is the difficulty to evaluate response to chemotherapy since computed tomography (CT) or positron emission tomography (PET)/CT often do not sufficiently show tumor spread. One ongoing phase Ⅱ trial in Germany addressing perioperative chemotherapy is the COMBATAC trial (multimodality treatment including neoadjuvant and adjuvant chemotherapy with cetuximab and CRS and HIPEC).

ORGAN PRESERVING CYTOREDUCTIVE SURGERY

Radical cytoreduction is many times associated with multivisceral resection because of diffuse organ infiltration. When performing cytoreductive surgery, the surgeon should, however, aim for preserving as many organs as possible. Moreover, the surgeon should leave as much as possible behind but without any oncological compromise. This approach seems quite unfamiliar to surgeons who do not deal with peritoneal metastases. In many cases, the small/large bowel can be preserved when addressed with patience for meticulous tumor resection since tumor nodules are mainly located on the peritoneal surface and can be removed without opening the bowel in most cases unless there is infiltrative growth.

From an oncologic point of view, a radical oncologic colon resection, except in primary colorectal cancer with peritoneal spread is not necessary in our opinion.

The surgical expertise should ideally include a broad surgical spectrum especially colorectal surgery. One technical challenge is certainly the liver hilus with the sulcus rex, sulcus arancii and segment 1 region which is very demanding to dissect with the risk of biliary or vascular damage when a certain experience in liver surgery is helpful.

SECOND LOOK SURGERY

Peritoneal carcinomatosis index (PCI), representing intraabdominal tumor load is a prognostic factor for survival. The lower the PCI, the better the prognosis maybe also due to the fact that a complete cytoreduction becomes more likely. Clinical signs of peritoneal metastases are often not specific and current imaging methods often do not detect small tumor nodules[18,19]. Given these findings, a second look protocol with a re-laparotomy within one year of colorectal surgery in high risk patients was proposed. The high risk patient for developing PSM suffers from either a perforated tumor or a local peritoneal spread at the time of primary surgery. Current data revealed quite a high percentage of PSM in those patients.

The second look protocol was firstly described by Elias *et al*[20]. Predicting the development of PSM in high risk patients is certainly a mile stone in the treatment of peritoneal metastases[20]. Although this approach is proactive, it may further prolong survival in those patients.

The administration of HIPEC even in a patient without macroscopic peritoneal disease needs further to be elucidated in randomized trials but seems to be promising so far. The "ProphyloCHIP" trial (Trial Comparing Simple Follow-up to Exploratory Laparotomy Plus "in Principle" HIPEC in Colorectal Patients) run by Prof Elias is addressing this particular point. In this randomised phase Ⅲ trial, colorectal cancer patients at risk to develop PC receive standard adjuvant chemotherapy after curative resection. After having excluded recurrent disease within 6 mo of follow-up they are randomised to either surveillance alone or explorative laparotomy and HIPEC. With this proactive approach, disease free and overall survival may be increased.

CONCLUSION

With newer imaging modalities such as PET/CT and PET/magnetic resonance tomography a better location of the tumor may be realized in future. Pro-active second look surgery, as far as there is no optimal imaging method, realizes the anticipation of diffuse peritoneal spread.

Making "unresectable patients" resectable is one challenging goal of neoadjuvant chemotherapy protocols in the future.

Using new protocols including intraperitoneal antibodies or even intraperitoneal virotherapy in patients with unresectable disease may further improve results.

Lastly and most importantly, a dedicated surgeon, an experienced anaesthesiologist and cooperating medical oncologists are mandatory to achieve excellent results and develop new concept in the treatment of peritoneal metastases.

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FIELD OF VISION

c-Met in pancreatic cancer stem cells: Therapeutic implications

Marta Herreros-Villanueva, Aizpea Zubia-Olascoaga, Luis Bujanda

Marta Herreros-Villanueva, Division of Oncology Research, Department of Medicine, Schulze Center for Novel Therapeutics, Mayo Clinic, Rochester, MN 55905, United States
Aizpea Zubia-Olascoaga, IkerChem, Oncology Therapeutics Department, 20009 San Sebastian, Spain
Luis Bujanda, Department of Gastroenterology, Centro de Investigación Biomédica en Red en Enfermedades Hepáticas y Digestivas (CIBERehd), Donostia Hospital-Biodonostia Institute, University of the Basque Country, 20014 San Sebastian, Spain
Author contributions: Herreros-Villanueva M, Zubia-Olascoaga A and Bujanda L designed and wrote the review.
Correspondence to: Luis Bujanda, MD, PhD, Department of Gastroenterology, Centro de Investigación Biomédica en Red en Enfermedades Hepáticas y Digestivas (CIBERehd), Donostia Hospital-Biodonostia Institute, University of the Basque Country, 20014 San Sebastian, Spain. luis.bujanda@osakidetza.net
Telephone: +34-94-3007173 Fax: +34-94-3007065
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Abstract

Pancreatic cancer is the deadliest solid cancer and currently the fourth most frequent cause of cancer-related deaths. Emerging evidence suggests that cancer stem cells (CSCs) play a crucial role in the development and progression of this disease. The identification of CSC markers could lead to the development of new therapeutic targets. In this study, the authors explore the functional role of c-Met in pancreatic CSCs, by analyzing self-renewal with sphere assays and tumorigenicity capacity in NOD SCID mice. They concluded that c-Met is a novel marker for identifying pancreatic CSCs and c-Met^{high} in a higher tumorigenic cancer cell population. Inhibition of c-Met with XL184 blocks self-renewal capacity in pancreatic CSCs. In pancreatic tumors established in NOD SCID mice, c-Met inhibition slowed tumor growth and reduced the population of CSCs, along with preventing the development of metastases.

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Key words: Cancer stem cells; c-Met; Gemcitabine; Self-renewal; Tumorigenicity

Peer reviewers: Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of General Medicine 2nd Unit, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University Hospital of Pisa, University of Pisa, via Roma 67, 56124 Pisa, Italy; Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief of Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, 5901 E. Seventh Str., Long Beach, CA 90822, United States

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INVITED COMMENTARY ON HOT ARTICLES

Pancreatic adenocarcinoma is one of the most aggressive neoplasias and the fourth leading cause of cancer death in the United States, with 5 year survival being less than 5%[1]. Part of the reason for the fatal prognoses of patients with pancreatic adenocarcinoma is the lack of response to the available therapies. In addition to achieving early diagnosis, the identification of markers for prognosis and response to therapy is necessary for the development of specific targeting agents for the management of patients with pancreatic cancer[2].

Conventional chemotherapy is directed at tumor cells that have limited tumorigenic potential, instead of targeting the cancer stem cell population.

Cancer stem cells (CSCs) conform to a subpopulation of tumoral cells that contribute mainly to drug resistance; since they can self-renew indefinitely and are charac-

terized by their relative quiescence. These properties, together with the contribution of the epithelial to mesenchymal transition, allow them to avoid conventional chemotherapy induced-cell death, and thus patients often suffer from tumor relapse[3,4].

A better understanding and characterization of these cells will be a helpful tool in the future for designing new therapies, since the CSC hypothesis predicts that only therapies that efficiently eliminate the CSC fraction of a tumor are able to induce long-term response and halt tumor progression. Novel therapeutic strategies that could target pancreatic CSCs will allow us to induce drug sensitivity, the inhibition of invasion, and the metastasis of pancreatic cancer, which will ultimately yield better treatment outcomes[5].

Although there have many studies attempting to establish markers for these CSCs, there is no complete or defined panel for the identification of these cells. Isolated CSCs have been reported to show increased tumorigenicity, chemoresistance, stationary phase, and asymmetric division to differentiate into non-stem progeny[6,7]. Based on these characteristics, pancreatic CSCs are identified by flow cytometry using cell surface markers such as CD44, CD24, ESA, CD133, ALDH1 and low proteosome activity[8-13].

In this study, new data is presented about CSC characterization and possible related therapies that should be considered for pancreatic cancer clinical trials.

c-Met is a receptor of the tyrosine kinase family that acts as a proto-oncogene and is stimulated by hepatocyte growth factor to mediate motility, invasion, and metastasis[14]. The levels of c-Met are increased in pancreatic carcinoma where c-Met signaling induces growth and invasion. Some authors have reported c-Met as a stem cell marker in pancreatic tissue[15], as well as in other tissues and organs such as the brain and gastrointestinal tract where the molecule regulates stem cell proliferation and cell renewal[16,17].

In this study, the authors conclude that c-Met is a novel marker for pancreatic CSCs and they show the functional role of this molecule in pancreatic tumors.

Using xenografts (immunodeficient mice NOD-SCID) and injecting pancreatic cancer cells from patients, they show that there is a subpopulation of cells that express high levels of c-Met (c-Met^{high}), while another subpopulation expresses low levels or does not express this maker at all (c-Met^{low}). Part of the c-Met^{high} population also expresses other previously described CSCs makers such as CD44, CD24, CD133, and ALDH1, but compared with these other markers, c-Met^{high} is better at identifying higher tumorigenic cancer cell populations (when injecting the same number of cells, c-Met^{high} cells produce tumors in 35% of the mice, while CD133+ produces tumors in 16% and CD44+ in 25%).

The population of positive cells for both the markers c-Met and CD44 (c-Met^{high}CD44+) enhances the *in vivo* tumorigenicity compared with the population with CD44+ alone. These data together show that c-Met is an important and novel marker of pancreatic CSCs.

Using sphere formation assay, it can be shown that c-Met^{high} cells have a self-renewal capacity, as they can form tumorspheres while c-Met^{low} are unable to do so.

On the other hand, treatment with the chemical inhibitor XL184 (a small molecule kinase inhibitor for c-Met) or the inhibition of c-Met with a specific shRNA impairs sphere formation, leading to apoptosis and cell cycle arrest; showing that c-Met activity is required to maintain a CSC population.

XL184 inhibits tumor growth and reduces the number of CSCs in xenograft mice with subcutaneous tumors and in orthotopic models.

When compared with Gemcitabine, the effect produced by XL184 is different. Although both XL184 alone and Gemcitabine alone treatments can inhibit tumor growth during the period that the animal is receiving treatment, some days after the end of treatment, the tumor growth continues at the same rate as the control tumors. By contrast, when the treatments are combined (XL184 in combination with Gemcitabine), tumor growth is prevented for up 32 d following cessation of treatment. This effect is due to the cells that each treatment targets. While Gemcitabine treatment results in an increase of the c-Met^{high}CD44+ population (likely because these cells are resistant to cell death with this chemotherapy) c-Met inhibition with XL184 leads to a decrease in c-Met^{high}CD44+ cells. Combination treatment prevents the increase in the CSC population observed with Gemcitabine alone and also contributes to a decrease in c-Met^{high}CD44+ population, suggesting that XL184 targets the CSC population specifically. Additionally, using an intracardiac injection model with pancreatic cancer cells, the authors demonstrated that XL184 treatment prevents metastasis development.

A study by Li *et al*[5] contributes significantly to the investigation into the stemness of pancreatic cancer cells. The study in question shows that c-Met is a new human pancreatic CSC marker. The results demonstrate that, in addition to identifying the population of cells with a self-renewal capacity in a pancreatic tumor, c-Met is a molecule necessary for maintaining tumoral cell growth and has capacity for metastasize.

Previous experiments had characterized CSCs as EpCAM+, CD44+, CD24+, CD133, low proteosome activity, ALDH+ for pancreatic CSCs, and CD133+ CXCR4+ for CSCs with higher metastatic potential. The addition of the new marker c-Met, already identified in other types of tumor, directs research towards the development of therapies targeting this tyrosine kinase receptor. In addition to its role as a biomarker of pancreatic CSCs, the authors purpose a potential therapy, showing reduced tumor growth in xenograft mice. They also demonstrated that c-Met has an important role in metastasis development, and that this process could be blocked by targeting c-Met specifically with XL184 treatment. XL184 (Cabozantinib) is a small-molecule kinase inhibitor with potent activity toward c-Met and vascular endothelial growth

factor receptor 2, as well as a number of other receptor tyrosine kinases including rearranged during transfection, kinase receptor, axl receptor tyrosine kinase, and fms like tyrosine kinase 3[18].

The authors demonstrated that XL184 targeting c-Met could be a promising therapy in combination with Gemcitabine treatment for pancreatic cancer. This is a very important achievement, since pancreatic cancer has a fatal prognosis due to its resistance to the currently available chemotherapy and radiotherapy. Although Gemcitabine is the most common chemotherapy used in pancreatic cancer patients, it has been demonstrated that it may not be particularly useful due to the properties that CSCs confer to the tumor. Some studies have demonstrated that, after Gemcitabine treatment, the subpopulation of CSC CD133+ is enriched[11] and that pancreatic cancer cell lines can undergo epithelial to mesenchymal transition after this treatment, resulting in an increased population of CD44+CD24+ESA+ cells[19].

Li *et al*[5] showed that the combination treatment of XL184 and Gemcitabine is an effective therapy for pancreatic cancer treatment. As different studies have shown, using a combination of therapies that target CSCs and the non-tumorigenic population of pancreatic cancer cells, this neoplasia can be effectively treated.

Collectively, these data and other recently published studies concerning different tumors, suggest that c-Met is a promising CSC marker for pancreatic cancer and XL184 is effective at inhibiting tumor growth, angiogenesis, and metastasis, and both should be seriously considered for clinical trials in combination with other available chemotherapy.

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Magnets, children and the bowel: A dangerous attraction?

Anil Thomas George, Sandeep Motiwale

Anil Thomas George, Department of Physiology, St Mark's Hospital, London HA1 3UJ, United Kingdom
Anil Thomas George, Sandeep Motiwale, Department of Paediatric Surgery, Queen's Medical Center University Hospitals, Nottingham NG7 2UH, United Kingdom
Author contributions: George AT drafted and wrote the review paper; Motiwale S discussed the topic and corrected the paper.
Correspondence to: Dr. Anil Thomas George, Department of Physiology, St Mark's Hospital, Watford Road, Harrow, London HA1 3UJ, United Kingdom. anilthomasgeorge@hotmail.com
Telephone: +44-1803-654806 Fax: +44-1803-654996
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Abstract

Reports of magnet ingestion are increasing rapidly globally. However, multiple magnet ingestion, the subsequent potential complications and the importance of the early identification and proper management remain both under-recognized and underestimated. Published literature on such cases could possibly represent only the tip of an iceberg with press reports, web blogs and government documents highlighting further occurrence of many more such incidents. The increasing number of complications worldwide being reported secondary to magnet ingestion point not only to an acute lack of awareness about this condition among the medical profession but also among parents and carers who will be in most cases the first to pick up on magnet ingestion. There still seems to be no consensus on the management of magnet ingestion with several algorithms being proposed for management. Prevention of this condition remains a much better option than cure. Proper education and improved awareness among parents and carers and frontline medical staff is key in addressing this rapidly emerging problem. The goal of managing such cases of suspected magnet ingestion should be aimed at reducing delays between ingestion time, diagnosis time and intervention time.

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Key words: Multiple magnet ingestion; Children; Bowel injury; Fistulation; Necrosis

Peer reviewers: Martin D Zielinski, MD, Department of Trauma, Critical Care and General Surgery, Mayo Clinic, 1216 2nd St Sw, Rochester, MN 55902, United States; Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of General Medicine 2 Unit, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University Hospital of Pisa, University of Pisa, Via Roma 67, 56124 Pisa, Italy; Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, CA, 5901 E. Seventh Str., Long Beach, CA 90822, United States

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INVITED COMMENTARY ON HOT ARTICLES

Background

Ingestion of foreign bodies is a common clinical problem; the occurrence of which has been steadily increasing all over the world. More than three quarters of such cases occur in children[1]. Of particular concern is the diagnostic and management dilemma that is posed by the ingestion of magnetic elements. Ingestion of a confirmed single magnet by itself does not pose a problem because it behaves just as an isolated foreign body. The single magnet in most cases moves through the gut harmlessly and silently and usually gets expelled without complications[2,3]. However, the ingestion of multiple magnets or a single magnet along with another metallic piece poses a totally different challenge as these magnetic elements can get attracted to each other with forces up to 1300 G[4] and any intervening bowel wall between the attracted parts eventually undergoing pressure necrosis. Subsequent fistulization between bowel loops can remain silent until it

leaks and peritonitis intervenes.

Magnitude of the problem

The issues of foreign body ingestion have been well discussed in the literature. However, multiple magnet ingestion, the subsequent potential complications and the importance of the early identification and proper management remain both under-recognized and underestimated. Reports of multiple magnet ingestion and its complications have been steadily increasing over the past few years, with over 15 cases being reported in the literature over 7 mo in 2012 compared to 10 cases in 2010, and two cases per year about a decade ago[3,5-8]. Published literature on such cases may represent only the tip of an iceberg with press reports, web blogs and government documents highlighting further occurrence of many more such incidents[5]. The extent of the problem is highlighted as a total of 128 published cases across 18 countries assimilated in 2010 have now expanded to over 150 cases over 22 countries in 2012[6-10]. The majority of such cases have involved the ingestion of either two or three magnetic elements, although there is one reported case of nearly 100 pieces[11].

Initial reports of this condition more than a decade ago were mainly confined to infants and toddlers. Children with a variety of psychological conditions including autism, developmental delays, history of pica, schizoid characteristics, Angelman syndrome, behavioral problems, Beckwith-Wiedemann syndrome with developmental delays, 4p syndrome, congenital hydrocephalus, mental retardation, reactive attachment, and anxiety were thought to be at a higher risk for accidental ingestion[11]. Of interest was the fact that this group comprised < 15% of the total cases reported so far.

Presently, the incidence of this problem no longer remains confined to these groups. Recent reports suggest that multiple magnet ingestion seem to be occurring with increasing frequency in fully developed older age groups[5,11,12].

The origin of most of these magnetic elements has been traced to toys, either directly belonging to the child or to an elder sibling[9,11-13]. A possible cause for the increase of such cases is the easy availability of cheap toys that contain magnetic elements[14]. New-generation magnets are made of combinations containing iron, along with other rare earth elements including boron and neodymium, and such magnets tend to be nearly 10 times stronger than standard iron magnets. This has enabled the miniaturization of magnets for inclusion in various small toys[3,15]. In many of these toys, the magnetic elements are poorly embedded in plastic moulds from which they can easily become detached[16].

Diagnosis

The key to diagnosis is to obtain a reliable history of magnet ingestion. A credible history of ingestion is crucial in the early recognition and correct management of this condition. The lack of a documented history of ingestion in nearly half of the reported cases even among the older age groups is of concern[5]. Younger children or those with developmental delays may be hindered by their inability to communicate effectively to their parents due to their limited linguistic or developmental abilities. Older children may hesitate to inform parents due to a sense of guilt or embarrassment or a fear of the consequences[9,17]. This may have a direct bearing on the time interval between ingestion and intervention. This may be shorter if there is evidence of ingestion or longer when only the occurrence of bowel complications may highlight an underlying magnetic pathology. Time intervals between ingestion and intervention have varied from a few hours to a few months[5].

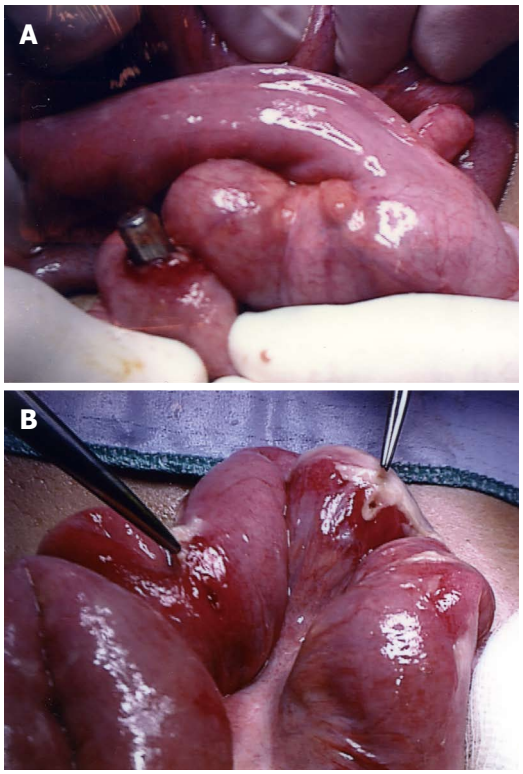


Figure 1 Symptoms may vary from totally asymptomatic or an unrelated pain to a mild flu-like illness with nonspecific symptoms of nausea, vomiting, cramps, or abdominal pain, to features of bowel obstruction or localized peritonitis. A: Magnetic cause for an acute appendicitis in an 8-year-old child; B: Laparotomy for peritonitis showed small bowel fistulation caused by two magnets.

Symptoms may vary from totally asymptomatic or an unrelated pain to a mild flu-like illness with nonspecific symptoms of nausea, vomiting, cramps, or abdominal pain to features of bowel obstruction or localized peritonitis[3,5,12,18] (Figure 1A, B).

Plain abdominal radiographs almost always pick up these objects and is a simple and quick screening test if a history of ingestion is obtained[19-21]. Plain radiography is a sensitive tool to screen and identify such cases but is poor in differentiating whether the ingested magnet is truly only single or is actually composed of multiple densely adherent magnetic elements. Although radiographs can be taken at different angles and planes, the

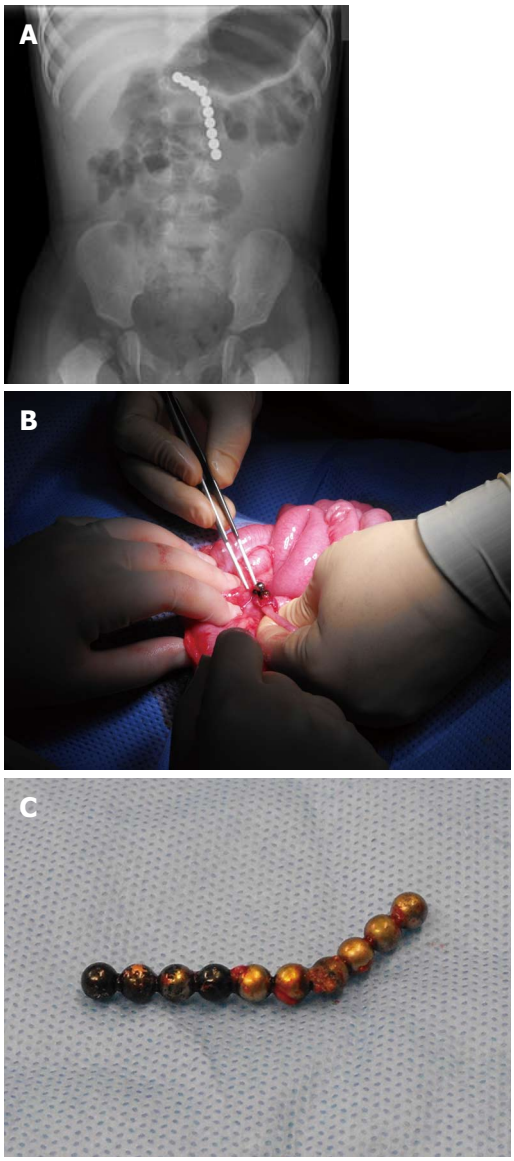


Figure 2 If multiple magnet ingestion is suspected, the entire gastrointestinal tract remains at risk of perforation even if the child is asymptomatic. A: Asymptomatic 18-mo-old child 96 h after ingestion of magnet; B: Silent gastro-jejeno-colic fistula at laparotomy; C: Ten strongly attracted magnetic balls lying across the fistula.

differences in radiographic appearances between a single magnet and multiple magnets adhered to each other may be subtle and impossible to differentiate[2]. Subtle separations or gaps between otherwise individual metallic pieces may point to the presence of multiple magnetic elements or the presence of intervening bowel between the magnets. However, this is by no means diagnostic and the absence of any gaps within the imaged magnet does not exclude more than one magnet nor the absence of bowel wall involvement[2]. In addition, the failure of the ingested magnetic element to progress through the bowel on subsequent follow-up radiographs should raise the suspicion of multiple magnetic elements with entrapped bowel, although this is not diagnostic because the multiple magnets can move *en bloc*[2,17,22,23]. Documenting the size and shape of the swallowed object on radiography and confirming the presence of only a single magnet may be challenging because such ingested magnets tend to be miniature and they usually originate from children's toys[3,12].

Computed tomography and ultrasound can be performed but may not contribute greatly because they generally lack the sensitivity to determine the multiplicity of or the presence of trapped bowel between the magnetic objects. Magnetic resonance imaging (MRI) scans should not be performed due to the magnetic nature of the ingested foreign body and bowel perforation secondary to inadvertent MRI has been reported[8].

Management

Management of ingested foreign bodies still relies to a great extent on "masterly inactivity", whereby the ingested foreign body traverses the gut and is expelled without any complications.

There still seems to be no consensus on the management of magnet ingestion, with several algorithms being proposed[2,3,5,21]. A common underlying theme is that in cases of multiple magnet ingestion, conservative management may have no appreciable role. Surgical exploration and removal remains the preferred management irrespective of the size or shape of the magnet[19].

A diagnostic and management dilemma arises if there is a doubt as to whether one or more magnets were ingested. A proper history is important to help identify between single or multiple magnet ingestion, but reliable documentation of ingestion may not always be present[5]. Clear differentiation is not always possible between the two because multiple magnets may tend to be densely adherent to each other and can mimic a single object on imaging.

Conservatively discharging the child back to the community without reliable evidence of single magnet ingestion may have the potential to cause unnecessary morbidity[3,5]. Undiagnosed multiple magnets can tend to remain asymptomatic for several weeks or months until potentially disastrous complications intervene[5].

Close observation of such cases even if they are asymptomatic may be prudent given the lack of any investigation which can effectively rule out multiple adherent magnets. If single magnet ingestion is suspected, normal progression through the bowel can be monitored closely with expulsion of the magnet through a bowel movement[21].

If multiple magnet ingestion is suspected, the entire gastrointestinal tract remains at risk of perforation even if the child is asymptomatic (Figure 2A-C). All such cases should be reviewed urgently by the surgeon with a view to magnet removal. If pediatric surgery expertise is not available, urgent transfer to an appropriate specialist center is important (Figure 3).

The aim of management in cases of suspected multiple magnet ingestion should be to shorten the delays between ingestion time, diagnosis time and intervention

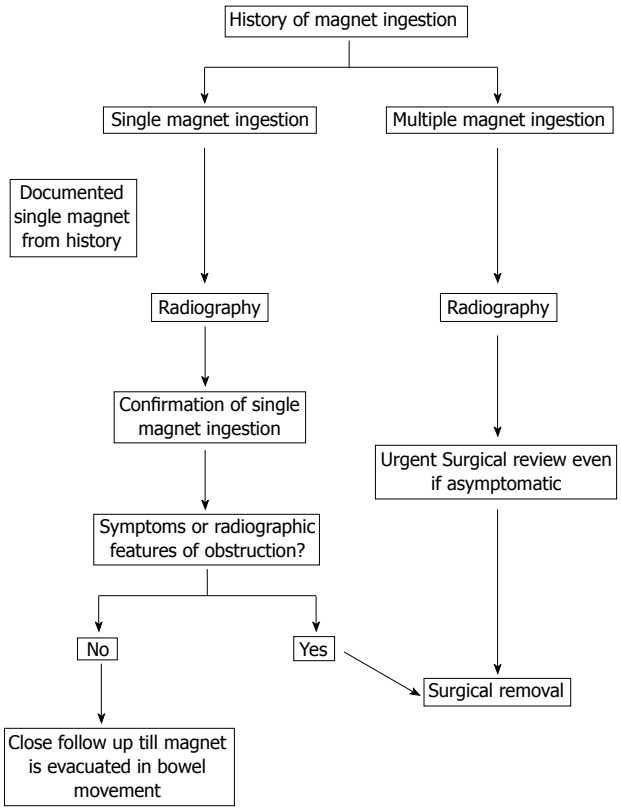


Figure 3 A view for magnet removal.

time.

Removal of the magnets is seminal and delaying the removal or waiting for evidence of bowel obstruction to develop may lead to unnecessary morbidity and even mortality[3,5]. All such reported cases with the exceptions of a few sporadic cases[11,18] have been managed with the surgical removal of the magnetic parts. Bowel injury following multiple magnet ingestion in a few conservatively managed cases may have been avoided possibly due to a near-simultaneous ingestion of the magnetic elements, which may have then behaved as a single large magnet. This however, would not be sufficient reason to recommend conservative management in such cases.

Removal of the ingested magnets can be retrieved endoscopically if they are in the esophagus, stomach or proximal duodenum[5]. Once the magnets move further into the small bowel, surgical removal either through an exploratory or a laparoscopic assisted laparotomy is required to localize and remove the magnets.

Field of vision

The field of vision regarding this condition is highly myopic. Prevention remains a much better option than cure for this rapidly increasing problem.

The increasing number of complications worldwide being reported secondary to magnet ingestion point not only to an acute lack of awareness about this condition among the medical profession but also among parents and carers who will be, in most cases, the first to pick up on magnet ingestion. Parents need to be alerted to the potential risk of silent bowel perforation and fistulation from accidental ingestion of magnets. The importance of increasing awareness regarding the potential complications of magnet ingestion is crucial. This could prompt parents and carers to identify earlier cases of suspected magnet ingestion and rapidly seek appropriate medical attention, and considerably reduce the delay between ingestion and diagnosis.

There also exists a lack of awareness among the medical profession about the potential of multiple magnet ingestion to do great harm. Improving awareness among frontline medical staff can help to reduce the time delay between ingestion and diagnosis, as well as between diagnosis and intervention.

There is also a need for tighter control and regulation of toys with magnetic components. Since 2006, there have been numerous alerts and recalls from Canadian and United States consumer product safety commissions issued in relation to children and the sale of toys with small ingestible magnetic parts[2,24]. The occurrence of such cases from over 21 countries worldwide highlights that this is no longer confined to a localized geographical region or population. Toy manufacturers all over the world can incorporate easily visible warnings regarding the presence of small magnetic parts in the toys on the labels. Highlighting age restriction on toys may not by itself cover much ground without improved awareness because younger children can accidentally ingest magnetic elements from toys that may have been appropriately bought for elder siblings in the family.

Parents, carers and medical staff globally remain under-informed and largely unaware regarding this rapidly increasing potential public health problem. The goal of managing such cases of suspected magnet ingestion should be to reduce the delays between ingestion time, diagnosis time and intervention time.

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TOPIC HIGHLIGHT

Cesare Ruffolo, MD, PhD, *Series Editor*

Laparoscopic distal pancreatectomy: Up-to-date and literature review

Maurizio Iacobone, Marilisa Citton, Donato Nitti

Maurizio Iacobone, Marilisa Citton, Donato Nitti, Minimally Invasive Endocrine Surgery Unit, Department of Surgical, Oncological and Gastroenterological Sciences, University of Padua, 35128 Padova, Italy
Author contributions: Iacobone M was responsible for conception and design, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, final approval of the version to be published; Citton M was responsible for conception and design, acquisition, analysis and interpretation of data, drafting the article and revising it, critically for important intellectual content, final approval of the version to be published; Nitti D was responsible for conception and design; analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, final approval of the version to be published.
Correspondence to: Marilisa Citton, MD, Minimally Invasive Endocrine Surgery Unit, Department of Surgical, Oncological and Gastroenterological Sciences, University of Padua, Via Giustiniani 2, 35128 Padova, Italy. marilisa.citton@libero.it
Telephone: +39-4-98211815 Fax: +39-4-98211378
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Abstract

Pancreatic surgery represents one of the most challenging areas in digestive surgery. In recent years, an increasing number of laparoscopic pancreatic procedures have been performed and laparoscopic distal pancreatectomy (LDP) has gained world-wide acceptance because it does not require anastomosis or other reconstruction. To date, English literature reports more than 300 papers focusing on LDP, but only 6% included more than 30 patients. Literature review confirms that LDP is a feasible and safe procedure in patients with benign or low grade malignancies. Decreased blood loss and morbidity, early recovery and shorter hospital stay may be the main advantages. Several concerns still exist for laparoscopic pancreatic adenocarcinoma excision. The individual surgeon determines the technical conduction of LDP, with or without spleen preservation; currently robotic pancreatic surgery has gained diffusion. Additional researches are necessary to determine the best technique to improve the procedure results.

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Key words: Pancreas resection; Laparoscopic distal pancreatectomy; Left pancreatectomy; Open pancreatectomy; Pancreatic fistula; Splenectomy; Spleen-preserving technique

Peer reviewers: Dr. Julio M Mayol, Digestive Surgery, Hospital Clinico San Carlos, Martin-Lagos S/n, 28040 Madrid, Spain; Eric S Hungness, MD, Assistant Professor, Department of Surgery, Northwestern University Feinberg School of Medicine, Chicago, IL 60611-2908, United States; Dr. Beat Schnüriger, Department of Visceral Surgery and Medicine, Bern University Hospital, 3010 Bern, Switzerland

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PANCREATIC SURGERY AND LAPAROSCOPY

Pancreatic surgery represents one of the most challenging areas in digestive surgery, and it has been historically associated with up to 50% morbidity and 5% mortality[1,2]. It is usually performed by open approaches, but following the increased experience in laparoscopic surgery of other districts and the availability of new technological devices, an increasing number of laparoscopic pancreatic procedures has been performed[3,4].

Laparoscopy has initially been used only for staging

pancreatic cancer. Cuschieri reported the first description of laparoscopic pancreatic resection in 1994[5]; few years later, Gagner published his initial experience with laparoscopic distal pancreatectomy (LDP) with spleen preservation including eight cases[6]. Actually, LDP has gained world-wide acceptance because the procedure does not require anastomosis or other reconstruction[7,8].

A comparison between open surgery and LDP confirms advantages commonly ascribed to minimal-access surgery such as reduced postoperative pain, faster recovery, fewer wound related and general morbidity[9-19]. Although the laparoscopic approach to distal pancreatectomy has become a feasible option over the last few years, it still faces two problems: firstly, sparing the spleen with or without ligation of the splenic vessels, and secondly, controlling the leak from the pancreatic remnant and pancreatic fistula[20]. However, some controversy about its indications and safety concerning long-term oncologic outcome, still exist[21-23].

Literature concerning LDP is relatively poor: Case reports, small case series and few multicentric larger studies have been published[21,24-26]. The aim of this paper is to review the most recent literature, in order to offer an up-to-date concerning the indications, the results and some technical controversial issues concerning LDP.

LAPAROSCOPIC DISTAL PANCREATECTOMY: LITERATURE REVIEW

A web search, focusing on humans, was performed by PubMed database, including papers published in the English language up to 20 November 2011, using the key words "laparoscopic distal pancreatectomy" or "left-side pancreatectomy". A total of 388 papers were found. The bibliographic research was further expanded considering the related references cited by the above-mentioned papers.

In order to avoid the confounding effect of case reports and small series, a more refined research, including series of at least 30 cases, was performed. Results published only in the abstract form were excluded; in case of multiple publications from the same authors or institutions, only the latest and largest series were considered, in order to avoid the duplication of cases.

Twenty-two papers, including 2016 operated patients were found when literature search was reviewed. The median number of cases included were 70 (range: 30-359). The results are summarized in the Table 1. Eighteen papers included retrospective series; patients were prospectively included only in four studies. Eight papers derived from multicentre group studies.

The review of the literature confirms that LDP may be considered a feasible and safe technique[21,27,28]. It represents more than 70% of the laparoscopic pancreatic resections actually performed[29]. However, the major part of the studies on LDP is represented by case series with a relatively small number of patients[23,30]; only 6% of papers includes more than 20 cases. Most of the studies have a retrospective design; subsequently, it is still difficult to trace any conclusion from the results of these experiences because of the insufficient level of evidence.

Several comparative studies have shown that the average operative time, blood loss, morbidity, mortality and length of hospital stay after laparoscopic access might favourably comparable with those after open surgery[9,11-19,29].

In particular Mehta *et al*[17] describes a tendency toward a shorter duration of surgery in laparoscopic resection compared to open, although without a significance level ($P = 0.071$).

By contrast, with these optimistic prospects, Baker has published a single-institution comparison between laparoscopic and open distal pancreatectomy, focusing on post-discharge readmission. The laparoscopic approach has been associated with a shorter hospital stay, but a higher rate of late readmission requiring interventional procedures[31]. Furthermore, LDP cannot be considered a routine laparoscopic procedure, since it requires an advanced technical laparoscopic skill.

To date, several aspects are still controversial, mainly related to the indications, the results of the procedure and some technical details.

INDICATIONS TO LAPAROSCOPIC DISTAL PANCREATECTOMY

The indications for LDP vary, depending on the study, although most operations of LDP have been performed because of benign lesions, neuroendocrine tumors or low-grade malignancies (in particular cystic tumors)[29,32]. However some cases of pancreatic adenocarcinoma have been reported[33]; the results of laparoscopic resection for left pancreatic adenocarcinoma are limited, and its safety for long-term oncologic outcome is strongly debated. This approach for the treatment of pancreatic carcinoma still requires prospective validation[34] (Table 2).

LDP has also been performed in patients with chronic pancreatitis[35]; laparoscopic necrosectomy for acute necrotizing pancreatitis has been also described[36]. Steering wheel injury typically involves pancreatic parenchyma in front of the vertebra; LDP preferably with spleen preservation, has been indicated for patients with pancreatic trauma[37-39].

Persistent hyperinsulinemic hypoglycemia of infancy is a rare disease due to focal islet cell adenomatosis that may cause severe neurogenic damage. LDP or enucleation of the focal lesion has been performed in pediatric patients, while an open near-total pancreatectomy has been indicated in an infant with sustained hypoglycemia[40,41].

Surgery is the only curative modality currently available for resectable pancreatic neuroendocrine tumors[26,42]. Spleen-preserving LDP is feasible and can be achieved in

Table 1 Laparoscopic distal pancreatectomy: Results of the literature review

Ref.	Year	Study type	*n*	Pathology
Butturini *et al*[98]	2011	Retrospective	43	SCN (14), MCN (9), SPT (4), NET (9), other (7)
Mekeel *et al*[99]	2011	Retrospective	34	SCN (11), NET (8), IPMN (6), MCN (4), other (5)
Nakamura *et al*[100]	2011	Retrospective	47	NET (9), MCN (10), IPMN (6), SPT (5), P (6), other (11)
Mehta *et al*[17]	2011	Prospective	30	NET (12), IPMN (4), ADK (7), other (7)
Song *et al*[101]	2011	Retrospective	359	SCN (51), MCN (72), SPT (52), NET (36), IPMN (76), ductal ADK (24), P (25), cyst (13), others (10)
Zerbi *et al*[102]	2011	Prospective	95	NET
Røsok *et al*[103]	2010	Retrospective	117	NET (53), carcinoma (28), metastases (5), cystic tumours (48), other (37)
Vijan *et al*[12]	2010	Retrospective	100	Cystic benign neoplasm (49), solid benign neoplasm (27), malignant neoplasm (20), other (4)
Jayaraman *et al*[104]	2010	Retrospective	107	NA
DiNorcia *et al*[16]	2010	Retrospective	95	NA
Ziegler *et al*[105]	2010	Retrospective	126	NA
Giulianotti *et al*[80]	2010	Retrospective	46	ADK (6), NET (carcinoma) (5), benign neoplasm (28), other (6)
Finan *et al*[13]	2009	Retrospective	50	ADK (6), MCN (9), SCA (9), IPMN (4), NET (9), other (13)
Weber *et al*[106]	2009	Retrospective	219	MCN (45), SCN (29), IPMN (23), cyst (14), SPT (5), NET (45), ADK (16), metastases (9), P (14), other (19)
Yoon *et al*[107]	2009	Retrospective	35	SPT (7), IPMN (4), MCN (4), NET (4), other (16)
Kooby *et al*[27]	2008	Retrospective	159	Cystic neoplasm (85), solid neoplasm (60), P (14), benign neoplasm (102), ADK (16)
Taylor *et al*[108]	2008	Retrospective	46	SCN (10), ductal ADK (9), MCN (6), other (19)
Laxa *et al*[109]	2008	Retrospective	32	SCN (10), NET (6), mucinous neoplasm (4), IPMN (4), other (8)
Sa Cunha *et al*[7]	2008	Prospective	37	NA
Melotti *et al*[110]	2007	Prospective	58	MCN (19), SCN (13), NET (9), SPT (5), ductal ADK (5), other (7)
Fernández-Cruz *et al*[28]	2007	Retrospective	82	Inflammatory tumor (8), cystic neoplasm (29), IPMN (10), NET (22), ductal ADK (13)
Mabrut *et al*[21]	2005	Retrospective	99	NA

n: Number of patients; SCN: Serous cystic neoplasm; MCN: Mucinous cystic neoplasm; ADK: Adenocarcinoma; SPT: Solid pseudopapillary tumor; IPMN: Intraductal papillary mucinous neoplasm; NA: Not available; NET: Neuroendocrine tumor; P: Pancreatitis. Number of case for each pathology are report between parenthesis.

Table 2 Indications to laparoscopic distal pancreatectomy

Benign disease	Borderline disease	Malignant disease
Acute/chronic pancreatitis	Neuroendocrine tumor	Invasive carcinoma
Trauma	Mucinous cystic neoplasm	Metastatic renal cell carcinoma
Persistent hyperinsulinemic hypoglycemia of infancy	Intraductal papillary mucinous neoplasm	
Serous cystic neoplasms		
Transplantation in the living donor		

most cases; it is indicated for insulinomas of the pancreatic body and tail. Intraoperative laparoscopic ultrasonography is essential to localize the tumor and to evaluate the gland for additional tumors. If the insulinoma is benign, solitary, and not close to the main duct, a laparoscopic enucleation of the neoplasm may be indicated[25,43,44].

Laparoscopic resections have been also performed in some patients with gastrinoma, VIPoma, glucagonoma[45], pancreatic polypeptidoma[46], or other islet neoplasms including so-called non-functioning tumors[6]. However, some of these neuroendocrine neoplasms are often malignant and conversion to open surgery is necessary in cases of gastrinomas and VIPomas complicated by lymph node metastasis[6,47,48].

In case of potentially malignant neuroendocrine neoplasms in the pancreatic body/tail, LDP might be indicated, but laparoscopic en bloc splenectomy with resection of the spleen vessels and regional lymph-nodes dissection are recommended[4]. In case of multiple endocrine neoplasia-1 (MEN-1) patients, multiple neoplasms are common (especially gastrinomas); in these cases tumors are also located at gastroduodenal sites; the intraoperative localization of the tumors by laparoscopic approach, is not always possible even by the laparoscopic ultrasound; subsequently it is not usually indicated[48]. Furthermore, since a prolonged postoperative follow-up (at least 10 years) is required in case of potentially malignant neuroendocrine tumors, consistent data concerning the cure and recurrences rate, are still not available.

Pancreatic mucinous cystic neoplasm (MCN) represents a further possible indication to LDP. It groups a spectrum of lesions ranging from benign mucinous cystadenoma to mucinous cystadenocarcinoma. MCN is characterized by a distinct ovarian type stroma; patients with these lesions are usually relatively young women, with the peak around in the fifth decade of life. These neoplasms are more common in the body/tail of pancreas and a complete resection of the lesion is indicated. In patients with MCN several authors[49,50] have reported successful treatments by LDP.

The pancreatic serous cystic neoplasms are usually benign cystic neoplasms. When serous cystic neoplasms are symptomatic or when a differential diagnosis from potentially malignant cystic neoplasm is not possible,

a resection (possibly LDP) is indicated[49-52]. In a large comparative study between open and LDP, cystic lesions represented 59% of the laparoscopically resected tumors and 46% of the tumor excised by a laparotomic approach[19].

Intraductal papillary mucinous neoplasms (IPMN), non-invasive mucin-producing, predominantly papillary, or rarely flat epithelial neoplasms arising from the main pancreatic duct (MD-IPMN) or its secondary branches (BD-IPMN), are grossly visible[53]. They involve the head of the pancreas more commonly than the body/tail and they affect older patients with the peak age in the seventh decade. For IPMN in the body/tail of the pancreas, LDP has been performed, but one should be aware that some IPMN is associated with invasive carcinoma, as it is for MCN[53,54].

LDP has also been carried out in patients with pancreatic invasive carcinoma[23], although the questions about the oncological consequences of laparoscopic pancreatic surgery remain strongly controversial[21].

The positive margin resection rate in pancreatectomy for ductal adenocarcinoma is difficult to understand because there is no defined standard for histologic margin assessment. A recent review of the large randomized trials highlight that positive margin rates ranged from 0% to 83%[55].

A prospective observational study comparing open *vs* LDP has shown that the number of lymph nodes removed during the laparoscopic procedure was significantly inferior in comparison to the open approach[10]. Another recent analysis from a multicentre group, has compared the results of laparoscopic and open distal pancreatectomy applied to pancreatic ductal carcinoma. Cancer outcomes in short-term (lymph nodes harvest and margin status) and long-term (survival) were found to be similar in both groups[32,33]. Finally, LDP have been sporadically described also for metastatic renal cell carcinoma[56] and for pancreas transplantation in the living donors[57,58].

SURGICAL TECHNIQUES

The individual surgeon determines the technical conduction of LDP; it is usually performed in a supine or in a right lateral position[59]. However, several technical variants may be used, and some controversies still exist. The main controversial aspects in LDP are related to the preservation of the spleen, by the number and location of orifices needed for approaching the pancreas, the extent of the resection and the technique used for the parenchymal transection.

Traditionally, distal pancreatectomy has been performed with splenectomy. However the spleen plays an important role in the immune system and spleen-preserving distal pancreatectomy is preferable, in patients with benign diseases or non-invasive neoplasms[8,21,51,60].

The rate of splenic conservation of LDP is reported to be between 32% and 84%[14,17,42]. Some comparative studies have assessed the outcomes preoperative intent of splenic conservation in distal pancreatectomies performed by laparoscopic and open approaches, with a higher success rate of preservation in the first group[14,17]. This is surely due to the better vision afforded by the magnification, used in laparoscopy.

Preservation of the spleen with distal pancreatectomy can be undertaken either with preservation or with sectioning of the splenic vessels by maintaining the blood flow to the spleen *via* short gastric vessels (technique of Warshaw[61]). The latter method is associated with a shorter operation time, less blood loss, and a shorter hospitalization. The subsequent appearance of gastric varices is a consequence of loss of the splenic vein but no bleeding from these collaterals during long-term follow up, has been described. However, a splenic infarction after the laparoscopic procedure of Warshaw[62] has been documented in several case reports[63].

A technical difficulty during the preservation of splenic vessels is the division of numerous shorts tributaries from the splenic vein spreading toward the pancreatic body/tail, that requires special caution[64]. The appropriate usage of modern technologies (electro thermal bipolar vessel sealer, ultrasonic coagulating shears) can achieve secure haemostasis of tributaries from splenic vessels.

The hand-assisted laparoscopic surgical techniques have been utilized in LDP, in order to facilitate the splenic vessel preservation, because incidental bleeding can be immediately stopped by finger compression, and in large cystic tumors for a safe mobilization of the tumor and adjacent tissue. Hand ports for the insertion of operator's left hand are placed through an upper midline incision, right subcostal incision, or right lower-quadrant transverse incision according to the preference of surgeons[65-68].

Single incision laparoscopic surgery has gained attention for its minimal invasiveness and aesthetic results. This approach has been commonly described for cholecystectomy and appendectomy[69-71]; recently it has also been reported for LDP[72]. It may be effective as conventional laparoscopic pancreatectomy, when performed by expert hands although it is still a challenging procedure[58]. Further studies are necessary to determine the advantages of this procedure in comparison with standard laparoscopy.

Even though laparoscopic surgery of the pancreas remains a very challenging technique, the classically available instruments have some relevant limits. Today, following the increasing use of the robotic surgery in other fields of general surgery, some robot-assisted pancreatic resections have been reported[73-75]. Robotic surgery, can bridge the gap between minimally invasive surgery and complex pancreatic surgery, thus extending the indications for minimally invasive pancreatic surgery.

Robot-assisted surgery increases the degrees of freedom of forceps manipulation and yields three-dimensional images[22,73,76-79]. It is a procedure with some technical

and oncological advantages over other minimally invasive techniques for distal pancreatic tumors, due to the stability of the operative field, the 3D, magnified vision, and the articulated robotic arms. Moreover, the robotic articulated arms permit a superior handling of vascular structures and articulated instruments minimizing manipulation of the pancreatic gland. This technique minimizes the risk of pancreatic capsule rupture as well as tumor cell dissemination, respecting oncological surgical standards and it could provide an increased chance for spleen preservation. Giulianotti *et al*[80] has highlighted that robot-assisted laparoscopic pancreatic surgery achieves complication and mortality rates comparable to open surgery approaches, but offers the advantages of minimally invasive surgery. However, robotic surgery has high costs especially concerning the installation and the operation time, which is longer than open surgery; at the same time, it also needs an adequate learning curve.

The extent of a resection in LDP is another controversial topic. It varies depending on the pathology. For example, when a non-invasive MCN is located in the tail of the pancreas, the gland can be divided to the right of the cystic lesion with a minimal margin and only the tail of the pancreas removed. For chronic pancreatitis, it is typically divided at the pancreatic neck anterior to the superior mesenteric vein[4,35]. Recently, pancreatic surgeons have performed parenchyma-sparing resections more frequently in order to decrease the rate of postoperative pancreatic insufficiency. Oncological radicality is essential and extended resections may be necessary in the setting of IPMNs, which encompass a spectrum of lesions from adenoma to invasive carcinoma.

Intraoperative examination of the transection margin is of paramount importance in the management of MD-IPMNs[81-83]. The International Association of Pancreatology guidelines for the management of IPMNs suggest that when adenoma or low-grade pancreatic intraepithelial neoplasia is found intraoperatively in a resection margin, no further resection is needed. In case of borderline neoplasms, high-grade dysplasia or invasive carcinoma, an extension of the surgical resection to a negative margin, requires total pancreatectomy[53].

MORBIDITY

The most frequent complications after distal pancreatectomy are the fistula formation and collection[21,84,85]; they are usually related to pancreatic parenchymal transection techniques, that is another controversial topic.

In 2005 the International Study Group on Pancreatic Fistula Definition consensus paper defined a postoperative pancreatic fistula as the existence of any fluid output after postoperative day three with amylase content greater than three times the upper normal serum value[86].

Mabrut reviewed a total of 897 patients who underwent open distal pancreatectomy and reported the incidence of pancreatic fistula to be 3.5%-26% (average 13%)[21]. The incidence of pancreatic fistula with laparoscopy in studies that involved at least ten patients ranged from 0% to 27%[85].

Various risk factors for fistula formation have been reported after distal pancreatectomy. It is likely to occur in a pancreas with a soft texture[21,84,85,87], and when a selective identification and ligation of the main pancreatic duct has not been performed[87-89].

Some authors have suggested that the selective ligation may be more difficult during laparoscopy and may contribute to increased fistula rates[21]. Nevertheless, comparative studies showed that the laparoscopic approach results in a similar rate of fistula formation than the open approach[14,15,17]. A meta-analysis of studies comparing minimally invasive (laparoscopic or laparoscopically assisted) to the open approach, showed a lower rate of pancreatic fistula formation for minimally invasive approach[11]. However these results need cautious interpretation, because they depend on the study by Kooby, that relied on a definition different from that of the other studies[27].

The debate regarding the technique of stump closure after distal pancreatectomy continues. All approaches, including fibrin glue, sealants, patches, stapler closure, electrocautery and suture have been tested in numerous studies[90,91].

The distal pancreatectomy trial included 352 patients that were randomly assigned to stapler or hand-sewn closure of the pancreatic remnant: both groups showed equal fistula rates of 30% and 36%[92].

The main duct ligation and parenchymal transection during LDP is most commonly performed using endoscopic linear staplers. The surgeon may oversew the staple line to ensure pancreatic ductal closure and haemostasis. In such cases, ultrasonic coagulating shears can be used, usually followed by a monofilament suture to secure the closure of the main pancreatic duct[21,63,93,94].

In a recent study, Sartori *et al*[95]describes a new technique of pancreatic transection by the electrothermal bipolar vessels sealer, which seals vessels and other tubular structures by reforming parietal collagen and elastin, particularly suitable for laparoscopic left pancreatectomy; but a possible suitability in terms of fistula reduction is still under investigation.

When using the hand assisted laparoscopic technique, the pancreas can be divided through the hand port wound as for conventional open surgery.

To prevent post-operative fistula, octreotide and its analogues, have also been used since 1990. However, despite twenty years of clinical use and performance in numerous studies, a recent Cochrane meta-analysis concluded that evidence is still lacking to give clear recommendations[96].

Intraabdominal drains are commonly used in most centres after pancreatic resections. There is no evidence that persisting drainage of postoperative wound fluid has a positive effect in avoiding fistulae; on the contrary, a recent study sustains that drains kept in situ for more than three days enhance fistula development[97].

The management of postoperative fistula remains a therapeutic challenge. Depending on patient's clinical conditions, it ranges from persisting drainage without any further measures, up to revision surgery. However in literature, after LDP, a conservative management of pancreatic fistula is usually described[85,87].

In conclusion, LDP is a feasible and safe procedure in patient with benign or low grade malignancies. Decreased blood loss and morbidity, early recovery and shorter hospital stay may be the main advantages. The introduction in particular of robotic surgery, can bridge the gap between minimally invasive surgery and complex pancreatic surgery. On the other hand, additional researches are necessary to determine the best technique for minimizing pancreatic fistula formation and to improve the results of procedure.

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REVIEW

Theoretical basis of a beneficial role for vitamin D in viral hepatitis

Khanh vinh quốc Lương, Lan Thi Hoàng Nguyễn

Khanh vinh quốc Lương, Lan Thi Hoàng Nguyễn, Vietnamese American Medical Research Foundation, Westminster, CA 92683, United States
Author contributions: Lương KVQ wrote the paper work; Nguyễn LTH designed research and searched for references.
Correspondence to: Khanh vinh quốc Lương, MD, Vietnamese American Medical Research Foundation, 14971 Brookhurst St.,Westminster, CA 92683, United States. lng2687765@aol.com
Telephone: +1-714-8395898 Fax: +1-714-8395989
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Abstract

Abnormal bone metabolism and dysfunction of the calcium-parathyroid hormone-vitamin D axis have been reported in patients with viral hepatitis. Some studies suggested a relationship between vitamin D and viral hepatitis. Genetic studies have provided an opportunity to identify the proteins that link vitamin D to the pathology of viral hepatitis (i.e., the major histocompatibility complex class Ⅱ molecules, the vitamin D receptor, cytochrome P_{450}, the renin-angiotensin system, apolipoprotein E, liver X receptor, toll-like receptor, and the proteins regulated by the Sp1 promoter gene). Vitamin D also exerts its effects on viral hepatitis *via* non-genomic factors, i.e., matrix metalloproteinase, endothelial vascular growth factor, prostaglandins, cyclooxygenase-2, and oxidative stress. In conclusion, vitamin D could have a beneficial role in viral hepatitis. Calcitriol is best used for viral hepatitis because it is the active form of the vitamin D_3 metabolite.

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Key words: Calcitriol; Hepatitis; Hepatitis B virus; Hepatitis C virus; Vitamin D

Peer reviewer: Dr. Roberto J Carvalho-Filho, MD, PhD, Department of Gastroenterology, Federal University of Sao Paulo, Rua Botucatu, 740, Vila Clementino, Sao Paulo 04023-060, Brazil

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INTRODUCTION

Abnormal bone metabolism and dysfunction of the calcium-parathyroid hormone (PTH)-vitamin D axis have been reported in patients with viral hepatitis. In these patients, bone mineral density (BMD) was reduced in the lumbar spine and femoral neck[1-4]. The prevalence and severity of bone loss increases based on the severity of the liver disease[2]. Biochemical markers of bone resorption, such as urinary telopeptide (NTX) and pyridinoline, bone-specific alkaline phosphatase, and serum levels of PTH, were increased in patients with chronic viral hepatitis[1,4-9]. Serum insulin-like growth factor-1 (IGF-1) and 25-hydroxyvitamin D_3 (25OHD) were lower in patients with viral hepatitis[1,8-10]. However, other studies demonstrated contradictory results with respect to bone metabolism in patients with chronic viral hepatitis. Osteosclerosis was reported in patients with hepatitis C virus (HCV) and was associated with normal levels of IGF-1. It is also associated with an increased levels of osteoproterin (OPG) and the ligand for receptor activator of nuclear factor-κB (RANK)[11,12]. Serum levels of PTH were lower in patients with HCV compared to controls[6,13]. These findings suggested that there might be a relationship between vitamin D and viral hepatitis. In this paper, we review the role of vitamin D in patients with viral hepatitis.

GENETIC FACTORS RELATED TO VITAMIN D IN VIRAL HEPATITIS

The major histocompatibility complex (MHC) class Ⅱ molecules play an important role in immune functioning

and are essential to the body's defense against infection. The human MHC class Ⅱ is encoded by three different isotypes, *HLA-DR*, *HLA-DQ*, and *HLA-DP*. Studies have suggested that several genes in the MHC region promote susceptibility to viral hepatitis. Human leukocyte antigen (HLA) genes, which are located in the MHC region, have been implicated in viral hepatitis susceptibility. *HLA-DRB1*12* is significantly more common in children with autoimmune hepatitis with positive hepatitis A IgM than in children with negative hepatitis A IgM[14]. In addition, the *HLA-DPA1* and *HLA-DPB1* genes are known to be associated with hepatitis B virus (HBV) infection in Han Chinese, Japanese, and Thai populations[15-18]. However, *HLA-DPA1* was not associated with the development of cirrhosis or hepatocellular carcinoma (HCC) in Han Chinese populations[19]. Genetic variants in the *HLA-DPA1* region may also affect treatment-induced hepatitis B e antigen (HBeAg) sero-conversion[20]. In the normal human liver, mRNA expression of *HLA-DPA1* and *HLA-DPB1* are important for control of HBV[21]. *HLA-DRB1*1101* correlates with less severe hepatitis in Taiwanese male carriers of HBV[22]. *HLA-DRB1*1302* was reported to be associated with protection against persistent HBV infection in Gambian populations[23]. In South Indian populations, a significantly higher frequency of *HLA-DRB1*0701* was observed in patients with chronic viral illness compared with individuals who spontaneously recover (SR), but *HLA-DRB1*0301* was noted to be of higher frequency in the SR group than the chronic HBV group[24]. In patients from Eastern Turkey, *DQ2* and *DQ8* have been noted to be markedly higher in patients with chronic HBV than those with SR[25]. The presence of *DQw1* may protect against chronic active HBV infection[26]. In addition, patients with chronic HBV infection and the *DQB1*0303* and *DRB1*08* haplotypes may be less responsive to interferon alpha (IFNα) treatment[27]. Moreover, *DRB1*11*, *DRB1*0301*, and *DRB1*04* were found to confer a significant protective advantage against HCV infection[28-31]. These alleles might be responsible for the selection of viral epitopes for presentation to $CD4^+$ T cells, leading to a more efficient immune response against the virus. In a meta-analysis study, both *DQB1*0301* and *DRB1*1101* were protective alleles and presented HCV epitopes more effectively to $CD4^+$ T lymphocytes than other epitopes, Indeed, subjects with these two alleles were at a lower risk of developing chronic HCV infection[32]. On the other hand, calcitriol is known to stimulate phagocytosis but suppresses MHC class Ⅱ antigen expression in human mononuclear phagocytes[33,34]. In peripheral blood leukocytes, the expression of *HLA-DR* decreased after calcitriol administration in renal transplant recipients[35]. Calcitriol also decreases interferon-gamma-induced HLA-DR antigen expression on normal and transformed human keratinocytes and cultured epithelial tumor cell lines[35,36]. Both DR and DQ protein levels on the surface of a myeloma cell line were decreased after calcitriol treatment[37]. Moreover, calcitriol inhibits the expression of all three subtypes of MHC class Ⅱ antigens (*HLA-DR*, *HL-ADP*, and *HLA-DQ*) as well as the accessory activity of monocytes, both in a dose- and time-dependent manner[38]. These findings suggest that calcitriol may have an impact on viral hepatitis by suppressing the expression of MHC class Ⅱ antigens.

Genetic studies provide an opportunity to link molecular variations with epidemiological data. DNA sequence variations, such as polymorphisms, exert both modest and subtle biological effects. Vitamin D exerts immunomodulatory and anti-proliferative effects through the vitamin D receptor (VDR) in numerous diseases. VDR gene polymorphisms are reported to be associated with distinct clinical phenotypes in Taiwanese hepatitis B virus (HBV) carriers[39]. There is an association between *Taq1* and *Fok1* polymorphisms of VDR and HBV outcomes in Chinese patients[40]. The *tt* genotype of VDR polymorphism is linked to persistent HBV infection in African patients[41]. Polymorphisms in the *TT* allele of exon 9 of VDR are associated with occult HBV infection in Iranian patients[42]. Significant differences in the frequency of the allelic distribution of the *Apa1* of VDR are reported to occur more frequently in patients with HBV complicated by severe liver disease as well as those with higher viral loads[43]. These observations suggest that alterations in VDR function may play a role in viral hepatitis.

The cytochrome P_{450} (*CYP*) system is responsible for the oxidation, peroxidation, and/or reduction of vitamins and for the metabolism of steroids, xenobiotics, and various drugs. The *CYP27B1-1260* promoter polymorphism has been reported to be associated with vitamin D deficiency and an increased risk of fracture in the elderly[44]. Reduced 25OHD levels associated with the *CYP27B1-1260* promoter polymorphism results in reduced 1,25OHD levels and are associated with failure to achieve sustained virologic response (SVR) in patients with hepatitis C virus (HCV) genotypes 1, 2, and 3[45]. In Huh7.5 hepatoma cells, HCV infection increased calcitriol production by inhibiting *CYP24A1* induction, the enzyme responsible for the first step in calcitriol catabolism[46]. *CYP24A1* methylation tended to correlate with better prognosis in HCV-related HCC[47].

The primary function of the renin-angiotensin system (RAS) is to maintain fluid homeostasis and regulate blood pressure. Angiotensin converting enzyme (ACE) is a key enzyme in the RAS and converts angiotensin (AT) I to the potent vasoconstrictor AT Ⅱ[48]. Hepatic stellate cells (HSCs) are recognized as the main collagen-producing cells in injured hepatic tissue. Angiotensin Ⅱ (AT Ⅱ) mediates key biological actions involved in hepatic tissue repair, including myofibroblast proliferation, infiltration of inflammatory cells, and collagen synthesis. Activated HSCs secrete AT Ⅱ[49]. ACE2 expression is significantly increased in the context of liver injury, in both humans and rats[50]. In addition, AT Ⅱ levels are much higher in patients with HBV when compared to controls. These levels were directly related to the severity of the illness and decrease markedly with captopril, which is an ACE inhibitor[51]. A statistically significant correlation has been

noted between polymorphisms in the promoter region of the *AT* gene and the development of progressive hepatic fibrosis in patients with chronic HCV[52]. In recurrent hepatitis C infection, male liver recipients who were carriers of the *D* allele of *ACE* appeared to gain more weight after liver transplantation; in female recipients, however, carriers of the *D* allele appear to experience more severe allograft fibrosis[53]. Losartan, an AT1 receptor blocker, attenuates liver fibrosis in experimental models and in patients with chronic hepatitis C and significantly decreases the expression of several profibrogenic and NADPH oxidase (NOX) genes[54]. The administration of AT-blocking agents reduced the development of graft fibrosis in hepatitis C recurrence after liver transplantation[55]. However, there is also an interaction between vitamin D and the RAS. The combination of ACE inhibitors with the ACE *DD* genotype has been shown to decrease the level of calcitriol[56]. In Turkish populations of hypertensive patients, the presence of the *ACE D* allele is associated with a higher risk of left ventricular mass index and ambulatory blood pressure measurement, which is negatively correlated with serum 25OHD levels[57]. In addition, genetic disruptions of the VDR gene result in overstimulation of the RAS, resulting in increased renin and AT Ⅱ productions and subsequently leading to elevated blood pressure and cardiac hypertrophy. Treatment with captopril reduced cardiac hypertrophy in VDR knockout mice[58], suggesting that calcitriol could function as an hormonal suppressor of renin biosynthesis. Moreover, calcitriol suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin core promoter[59] and decreases ACE activity in bovine endothelial cells[60].

Apolipoprotein E (ApoE) is critical to systemic and local lipid transport and is a major genetic factor in viral hepatitis. The hepatitis virus is associated with serum lipoproteins, including ApoE and ApoB, and may enter cells *via* the low-density lipoprotein receptor (LDL-R). In *in vitro* models, the co-culture of hepatocytes with liver sinusoidal endothelial cells (LSEC) significantly increases the ability of hepatocytes to uptake low-density lipoprotein (LDL) and also results in a high level of HCV-like particle uptake[61]. The cell surface expression of LDL-R has been reported to correlate well with LDL-cholesterol and HCV-viral load[62]. ApoE antibody can block both HCV entry an the knockdown of the LDL-R reduced HCV infection of cells[63]. Human ApoE is required for the infectivity and assembly of HCV[64,65]. The *ApoE ε4* allele protects against severe liver disease caused by HCV[66], while *ApoE ε3* is associated with persistent HCV infection[67]. In addition, patients with chronic hepatitis C who do not carrying an *ApoE* ε3 allele, as well as carriers of a single *ApoE* ε3 allele with a serum cholesterol concentration over 190 mg/dL, were more likely to have a favorable outcome[68]. Moreover, lipoprotein abnormalities found in the early phases of acute hepatitis; low levels of serum cholesterol and ApoA associated with the severity of liver cell injury in chronic liver disease[69]. The nonstructural protein 5A (NS5A) of the HCV has been shown to interact with $ApoA_1$[70]. A decreased level of $ApoA_1$ was found in the LDL fractions of HCV-infected patients; the specific siRNA-mediated down-regulation of $ApoA_1$ led to a reduction in both HCV RNA and viral particle levels in culture[71]. On the other hand, the *ApoE4* allele is reported to be associated with decreased bone mass in postmenopausal Japanese women[72]. The common *ApoE* polymorphism has a complex effect on bone metabolism in peri-menopausal Danish women: those with *ApoE2* have lower bone mineral losses in the femoral neck and hip region than other women, whereas those with *ApoE4* gain more bone mineral than other women[73]. Calcitriol has been shown to induce macrophages to exhibit specific saturable receptors for LDL and acetyl-LDL; the LDL receptor of 1,25OHD-induced macrophages has been found to exhibit specificity for ApoB and E-containing lipoproteins[74]. In ApoE knockout mice, an animal model of dyslipidemia, high oxidative stress, and pronounced atherosclerosis after unilateral nephrectomy, animals developed less plaque growth and calcification with vitamin D analog treatment (paricalcitol) compared to healthy controls[75,76]. *ApoE ε4*, however, is associated with higher serum 25OHD levels[77]. Moreover, hypovitaminosis D is associated with reductions in serum $ApoA_1$[78] and a highly significant positive correlation was found between serum concentrations of 25OHD and *$ApoA_1$*[79]. In addition, calcitriol was reported to suppress *$ApoA_1$* gene expression at the transcriptional level in hepatocytes[80].

Lipids have been shown to play important roles in the viral life cycle and pathogenesis of infection. HBV infection of primary hepatocyte cultures is dependent on the presence of cholesterol in the viral envelope. The extraction of cholesterol from HBV purified from the plasma of HBV-infected patients leads to a strongly reduced level of infection, whereas infectivity is only regained by adding cholesterol back[81]. A number of lipid metabolic pathways were disrupted by HCV infection, resulting in an increase in cholesterol and sphingolipid levels[82]. Higher serum triglycerides, total cholesterol and LDL levels were correlated with higher HCV RNA levels[83]. Ceestatin, a novel small molecule inhibitor of hepatitis C virus replication, inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in a dose-dependent manner[84]. Polyunsaturated liposomes are reported to be antiviral against hepatitis B and C viruses by decreasing cholesterol levels in infected cells[85]. Moreover, HCV and HBV X protein increases the hepatic lipogenesis is mediated predominantly by the liver X receptor (LXR)[86-88]. LDL receptor-related protein 5 (LRP5) is essential for normal cholesterol and glucose metabolism. Mice lacking LRP5 develop both increased plasma cholesterol levels when fed a high-fat diet markedly impaired glucose tolerance when fed a normal diet[89]. HCV core protein activates Wnt/β-catenin signaling molecules, such as LRP5/6 co-receptors[90], whereas calcitriol regulates the expression of LRP5 *via* DNA sequences elements located downstream of the transcription start site[91]. Notably,

high serum 25OHD concentrations are associated with a favorable serum lipid profile, e.g., total cholesterol and high-density cholesterol (HDL-C)[92]. Low levels of active vitamin D (calcitriol) are also associated with low HDL-C levels[93]. Moreover, calcitriol has been shown to suppress foam cell formation by reducing acetylated LDL (AcLDL) and oxidized LDL (oxLDL) cholesterol uptake by macrophages[94]. In addition, calcitriol also inhibits the activity of HMG-CoA reductase, an enzyme required for cholesterol biosynthesis[95]. In male VDR knockout mice, serum total cholesterol and LXRβ levels were significantly higher than those in wild type mice[96]. The crosstalk between LXRα and VDR signaling in the regulation of bile acid metabolism suggests a possible contribution of the VDR to the modulation of bile acid and cholesterol homeostasis[97].

Toll-like receptors (TLRs) are a group of glycoproteins that functions as surface trans-membrane receptors and are involved in innate immune responses to exogenous pathogenic microorganisms. Substantial evidence supports an important role for TLRs in the pathogenesis and outcomes of viral hepatitis. There is a correlation between hypo-responsiveness to TLR ligands and liver dysfunction in HCV infection[98]. The disruption of TLR-3, TLR-7, and TLR-9 signaling was reported in viral hepatitis[99-101]. *In vivo*, TLR signaling also inhibits HBV replication[102]. TLR-2 polymorphisms that impair the recognition of HCV core and nonstructure 3 proteins may be associated with allograft failure and mortality after liver transplantation for chronic HCV[103,104]. These polymorphisms affect HCV viral loads and increase the risk of HCC in patients infected with HCV genotype 1[105]. The TLR-3 polymorphism may predispose Asian Indian populations to HCV infection[106] and protect Han Chinese populations from HBV recurrence after liver transplantation[107]. TLR-7 polymorphisms are protective against from development of inflammation and fibrosis in male patients with chronic HCV infection and are predictive of the response to IFN treatment[108-110]. TLR-2 and TLR-4 polymorphisms are not associated with liver cirrhosis in HCV infected Korean patients[111]. RNA levels of TLRs 2, 4, 6, 7, 8, 9 and 10 were up-regulated in both the monocytes and T cells of HCV-infected patients when compared to controls[112,113]. In obese rats, vitamin D deficiency increases the expression of hepatic mRNA levels of TLR-2, TLR-4, and TLR-9[114]. However, calcitriol is also known to suppress the expression of the TLR-2 and TLR-4 protein and mRNA in human monocytes; it also triggers hypo-responsiveness to pathogen-associated molecular patterns[115]. Calcitriol has also been shown to down-regulate intracellular TLR-2, TLR-4 and TLR-9 expression in human monocytes[116]. TLR activation results in the expression of VDR and 1α-vitamin D hydroxylase in human monocytes[117]. Calcitriol can cause vitamin D-induced expression of cathelicidin in bronchial epithelial cells[118] and may enhance the production of cathelicidin LL-37[119]. The addition of a VDR antagonist has been shown to inhibit the induction of cathelicidin mRNA by more than 80%; consequently, the protein expression of this antimicrobial agent was reduced by approximately 70%[118].

The HBV major surface antigen promoter contains four functional transcription factor Sp1 binding sites, which likely contribute to the level of expression from this promoter during viral infection[120-122]. HCV-core protein functions as a positive regulator of IGF-Ⅱ transcription *via* the protein kinase C (PKC) pathway, and Sp1 and Egr1 are direct targets of the transcriptional regulation of the IGF-Ⅱ, which plays an important role in HCV pathogenesis during the formation of HCC[122,123]. Steatosis is an important clinical manifestation of HCV infection. Sp1 is involved in sterol regulatory element-binding protein-1c (SREBP-1c) activation, which activates the transcription of lipogenic genes by HCV-3a NSSA[124]. Moreover, Sp1 might participate in triggering HCV core protein up-regulation of the extracellular matrix metalloproteinase (MMP) inducer expression and progression of metastasis[125]. On the other hand, binding sites for the transcription factor Sp1 have been implicated in the hormone-dependent transcription of several genes. In cultured human fibroblasts, the level of CYP24 (25-OHD 24-hydroxylase) mRNA plays a key role in the metabolism of 1,25OHD and increases responsiveness to calcitriol by 20 000-fold. Two vitamin D-responsive elements (VDREs) located upstream of the *CYP24* gene are primarily responsible increased mRNA levels, and Sp1 has been noted to act synergistically with these VDREs in this induction[126]. The mVDR promoter is controlled by Sp1 sites[127] and functions as the transactivation component of the VDR/Sp1 complex to trigger gene expression[128]. Moreover, the genes encoding Sp1, VDR, the locus for the vitamin D-dependent rickets type Ⅰ, and hepatitis B virus-positive hepatocellular carcinomas from Thai patients were mapped to human chromosome 12q[129,130].

THE NON-GENETIC ROLE OF VITAMIN D IN HEPATITIS

A high prevalence of vitamin D deficiency was reported in HCV patients[10,131]. Low serum 25OHD levels are also found in patients with human immuno-deficient virus (HIV) and HCV and are correlated with severe liver fibrosis[132,133]. Preparations containing vitamin D_3 were shown to be effective in reducing the severity of the syndrome associated with osteo-arthropathy, including a decrease in BMD in Ukrainians with chronic hepatitis B and C[134]. The combination of vitamin A (25 000 IU) and vitamin D_2 (2500 IU) enhances the re-vaccination reaction against HBV in Chinese children[135]. *In vitro*, vitamin D_2 is reported to inhibit HCV RNA replication and its combination with β-carotene and linoleic acid also causes an additive and/or synergistic effect with respect to HCV RNA replication[136]. VDR mRNA and protein were found in the rat liver throughout the animal's life span[137]. In another study, however, human and mouse hepato-

cytes were found to have very low nuclear VDR (nVDR) mRNA and protein levels, whereas the sinusoidal endothelial, Kupffer, and stellate cells of the normal rat liver as well as a mouse biliary cell line clearly expressed the nVDR gene transcript[138]. Vitamin D_3 dramatically inhibits HCV production in Huh7.5 hepatoma cells and in combination with INF-α, also synergistically suppresses HCV production in human hepatocytes[47]. Serum vitamin D levels are complimentary to the IL-28B polymorphism in enhancing the accurate prediction of the SVR in patients undergoing treatment for chronic HCV[139]. Low vitamin D is linked to severe liver fibrosis and low SVR in response to IFN-based therapy in genotype 1 chronic HCV patients[10]. Vitamin D supplementation also improves SVR in chronic HCV-naïve patients[140] and in response to antiviral treatment for recurrent HCV infection in liver transplant patients[141]. These findings suggest that vitamin D may play a role in the treatment of HCV. Chronic infection with viral hepatitis is a major risk factor worldwide for the development of HCC. Vitamin D analogs have been reported to reduce tumor volume in patients with inoperable HCC[142] and to increase apoptosis of hepatocarcinoma cells by 21.4%[143]. In another pilot study, an intra-arterial injection of calcitriol in lipiodol into the hepatic artery was given to eight refractory HCC patients and led to the stabilization of α-fetoprotein levels[144].

MMPs are proteolytic enzymes that are responsible for extracellular matrix remodeling and the regulation of leukocyte migration through the extracellular matrix, which is important step for inflammatory processes and infectious diseases. MMPs are produced by many cell types including lymphocytes, granulocytes, astrocytes and activated macrophages. During the course of chronic HCV infection, hepatic mRNA expression of MMPs has been shown to either increase steadily with disease progression (MMP-1, MMP-2, MMP-7, and MMP-14) or increase transiently (MMP-9, MMP-11, and MMP-13), depending on the type of MMP[145]. Serum and tissue MMP-9 expression were reported to decrease in chronic HCV patients treated with pegylated INF-α2b and ribavirin[146]. The ratio of MMP-9 to MMP-2 is useful in distinguishing between patients with early stage and advanced HCC[147]. Serum TIMP-1 levels decreased significantly during and after treatment in sustained responders[148]. MMP-3 polymorphisms are associated with persistent HBV infection and advanced liver cirrhosis in Korean populations[149,150]. MMP-1, MMP-3, and MMP-9 polymorphisms are associated with the progression of HCV-related chronic liver disease in Japanese populations and may be a risk factor for poor prognosis in HCC patients[151,152]. However, VDR knock-out mice demonstrated an increased influx of inflammatory cells, phospho-acetylation of NF-κB associated with increased pro-inflammatory cells, and up-regulation of MMP-2, MMP-9, and MMP-12 in the lung[153]. The VDR *TaqI* polymorphism is associated with a decreased production of TIMP-1, which is a natural inhibitor of MMP-9[154]. Calcitriol modulates tissue MMP expression under experimental conditions[155], down-regulates MMP-9 levels in keratinocytes, and may attenuate the deleterious effects caused by the excessive TNF-α-induced proteolytic activity associated with cutaneous inflammation[156]. Calcitriol inhibits both basal and the staphylococcus-stimulated production of MMP-9 in human blood monocytes and alveolar macrophages[157]. Moreover, a vitamin D analog was also reported to reduce the expression of MMP-2, MMP-9, vascular endothelial growth factor (VEGF) and PTH-related peptide in Lewis lung carcinoma cells[158]. Furthermore, calcitriol significantly attenuated *Mycobacterium tuberculosis* (*M. tuberculosis*)-induced increases in the expression of MMP-7 and MMP-10, while suppressing the secretion of MMP-7 by *M. tuberculosis*-infected PBMCs. MMP-9 gene expression, secretion and activity were significantly inhibited, irrespective of infection status[159]. Calcitriol also suppressed the production of MMPs (MMP-7 and MMP-9) and enhanced the level of TIMP-1 in tuberculosis patients[160]. In human articular chondrocytes, calcitriol significantly suppresses the increased production of MMP-9 that is induced by phorbol myristate acetate (PMA)[161]. These studies suggest that calcitriol may play an important role in the pathological process of viral hepatitis by down-regulating the levels of MMPs and regulating the levels of TIMPs.

Angiogenesis is a complex process involving the coordinated steps of endothelial cell activation, proliferation, migration, tube formation and capillary sprouting, which require the participation of intracellular signaling pathways. VEGF is a key mediator of angiogenesis. Vascular changes associated with angiogenesis usually occur in cancer; however, they have also been reported to occur in inflammatory disease processes. HCV C protein can activate the expression of VEGF in hepatoma cell lines ($HepG_2$) and might contribute to viral carcinogenesis[162]. Co-expression of the HBV X gene and the HCV core gene also increase the expression of VEGF in $HepG_2$ cells and act synergistically in carcinogenesis[163]. The expression levels of TNFα mRNA and VEGF mRNA showed a positive correlation with the progression of viral hepatitis to cirrhosis, i.e., the higher levels of TNFα and VEGF mRNA, the higher the prevalence of HCC[164]. HBV X protein is known to up-regulate the expression of VEGF, thereby promoting angiogenesis in HCC *via* NFκB signaling pathway[165]. Serum VEGF concentration is a predictor of invasion and metastasis in HCC[166] and positively correlates with the recurrence rate of HCC after curative resection[167]. In contrast, calcitriol was reported to inhibit angiogenesis *in vitro* and *in vivo*[168]. Calcitriol significantly inhibits VEGF-induced endothelial cell spouting and elongation in a dose-dependent manner and decreases the production of VEGF[169]. Calcitriol is a potent inhibitor of retinal neovascularization in a mouse model of oxygen-induced ischemic retinopathy[170]. Vitamin D and its analog also reduce the expression of VEGF in various cancer cell lines[158,171]. Moreover, *DBP-maf* was reported to inhibit angiogenesis and tumor

growth in mice[172] and inhibits the VEGF signaling by decreasing VEGF-mediated phosphorylation of VEGF-2 and ERK1/2, a downstream target of the VEGF signaling cascade[173]. These findings suggested that vitamin D modulates angiogenesis in viral hepatitis and may impact the mechanism of progression to HCC in patients with viral hepatitis.

Prostaglandins (PGs) play a role in inflammatory processes. Cyclooxygenase (COX) participates in the conversion of arachidonic acid to PGs. HBV X protein was reported to up-regulate levels of COX-2, 5-lipoxygenase and phosphorylated extracellular signal-regulated protein kinase ½ (p-ERK1/2) and releases arachidonic acid metabolites in liver cells[174]. In liver samples from patients with chronic HCV infection, there is a significant correlation between the dominant intensity of COX-2 and the presence of histological steatosis and an inverse correlation was observed between COX-2 and viral load[175]. COX-2 up-regulates VEGF expression and tumor angiogenesis in HBV-associated HCC *via* PG production; selective COX-2 inhibitors may block HCC-associated angiogenesis and an increase in platelet counts when used with pegylated TFNα2a[176,177]. Indomethacin also cleared HBV DNA in chronic healthy carriers, and 5 patients with positive HBeAg became negative after 4 mo[178]. On the other hand, calcitriol has been reported to regulate the expression of several key genes involved in the PG pathway, resulting in a decrease in PG synthesis[179]. Calcitriol and its analogs have been shown to selectively inhibit the activity of COX-2[180]. These findings suggested that vitamin D plays a role in modulating the inflammatory process in viral hepatitis.

Reactive oxygen species (ROS) are produced by activated phagocytes as a part of their microbicidal activities. Intracellular hydrogen peroxide (H_2O_2) levels are significantly higher in patients with chronic HCV infection than in asymptomatic carriers and positively correlates with alanine amino-transferase (ALT) levels[181]. ROS can also modulate the intracellular level of HBV X protein. The direct addition of H_2O_2 to cells significantly increased the level of HBV X protein in HBV X protein ChangX-34 cells, while antioxidants completely abolished the increase in HBV X protein[182]. There is a significant decrease glutathione (GSH) levels in the patients with HBV-infected[183]. Superoxide dismutase (SOD) was present in peripheral blood mononuclear cells (PBMC) but was absent in the liver of patients with chronic HCV infection[184]. Levels of lipid peroxidation products are increased in serum, leukocyte, and liver specimens in HCV patients[185]. Similarly, calcitriol has been reported to exert a receptor-mediated effect on the secretion of H_2O_2 by human monocytes[186]. Human monocytes in culture gradually lose their capacity to produce superoxide when stimulated. The addition of calcitriol, lipopolysaccharide or lipoteichoic acid restored the ability of stimulated monocytes to produce superoxide and increased their oxidative capacity when compared with unstimulated monocytes[187]. Calcitriol can also protect nonmalignant prostate cells from oxidative stress-induced cell death by eliminating ROS-induced cellular injuries[188]. Vitamin D metabolites and vitamin D analogs were reported to induce lipoxygenase mRNA expression, lipoxygenase activity and ROS in a human bone cell line[189]. Vitamin D can also reduce the extent of lipid peroxidation and induce SOD activity in the hepatic anti-oxidant system of rats[190]. These findings suggested that vitamin D modulates oxidative stress in viral hepatitis.

Nitric oxide (NO) is a reactive nitrogen species (RNS) that is critical in the redox biology of hepatocytes and is formed by nitric oxide synthase (NOS). In the liver, iNOS was found to be important in the development and propagation of inflammation. Viral hepatitis is associated with an increased iNOS expression[191,192]. HCV infection can also stimulate the production of iNOS through the activation of the iNOS gene by the viral core protein and the NS3 protein[191]. In patients with HCC, the combined negative expression of iNOS and COX-2 on histology has a significant impact on patient survival[193]. Oxidative DNA damage has been reported to increase chromosomal aberrations associated with cell transformation, and oxidative stress has also been suggested in the development of HCV-associated HCC. Oxidative DNA damage was observed in circulating leukocytes and occurs as an early event in chronic HCV infection[194]. NO often damage mitochondria, leading to the induction of double-stranded DNA breaks and the accumulation of oxidative DNA damage[195]. The viral core and NS3 proteins were shown to be responsible for inhibition of DNA repair, which is mediated by NO and ROS[196]. On the other hand, the activation of macrophage 1α-hydroxylase results in an increase in 1,25 OHD, which inhibits iNOS expression and reduces the NO produced by LPS-stimulated macrophages[197]. This calcitriol production by macrophages could provide protection against the oxidative injuries caused by the NO burst. Calcitriol is known to inhibit LPS-induced immune activation in human endothelial cells[198]. Calcitriol enhances intracellular GSH pools and significantly reduces the nitrite production induced by LPS[199]. In human macrophage-like cells, calcitriol induces iNOS and suppresses the growth of *M. tuberculosis*[200]. Moreover, calcitriol protects against doxorubicin-induced chromosomal aberrations in rat bone marrow cells[201]. Calcitriol also acts synergistically with vanadium in inhibiting diethylnitrosamine-induced chromosomal aberrations and DNA-strand breaks in the rat liver[202]. In regenerating liver cells, calcitriol regulates the synthesis of DNA polymerase-alpha, generates functional ribonucleotide reductase subunits, and induces DNA replication[203,204]. In addition, calcitriol appears to be effective in suppressing liver-specific early chromosomal damage as well as DNA damage during the process of rat hepatocarcinogenesis[205].

CONCLUSION

The relationship between vitamin D and viral hepatitis

has been discussed. Vitamin D may have a beneficial role in viral hepatitis. Genetic studies have provided the opportunity to determine what proteins link vitamin D to the pathology of viral hepatitis. Vitamin D also exerts its effect on viral hepatitis *via* non-genomic mechanisms. As a result, it is imperative that vitamin D levels in patients with viral hepatitis be followed. Many studies use the relationship between serum PTH and 25OHD to define the normal range of serum 25OHD. According to the report on Dietary Reference Intakes for vitamin D and calcium by the Institute of Medicine (IOM), persons are at risk of deficiency at serum 25OHD levels less than 30 nmol/L. Recently, Saliba *et al*[206] suggested that a 25OHD threshold of 50 nmol/L is sufficient for PTH suppression and prevention of secondary hyperparathyroidism in persons with normal renal function. Calcitriol is best used for viral hepatitis, because of its active form of vitamin D_3 metabolite and inhibits inflammatory cytokine expression. Adjusting dose for calcitriol depends on serum calcium and PTH levels. However, monitoring of serum 25OHD after calcitriol intake is not necessary because calcitriol inhibits the production of serum 25OHD in the liver[207,208]. Further investigation with calcitriol in viral hepatitis is needed.

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ORIGINAL ARTICLE

Anti-inflammatory effects of Lacto-Wolfberry in a mouse model of experimental colitis

David Philippe, Viral Brahmbhatt, Francis Foata, Yen Saudan, Patrick Serrant, Stephanie Blum, Jalil Benyacoub, Karine Vidal

David Philippe, Viral Brahmbhatt, Francis Foata, Yen Saudan, Patrick Serrant, Stephanie Blum, Jalil Benyacoub, Karine Vidal, Department of Nutrition and Health, Nestlé Research Center, CH-1000 Lausanne, Switzerland

Author contributions: Philippe D, Blum S, Benyacoub J and Vidal K designed the experiments; Philippe D, Foata F, Saudan Y and Serrant P performed the research; Philippe D, Brahmbhatt V, Benyacoub J and Vidal K analyzed the data and wrote the manuscript; all authors approved the final manuscript.

Correspondence to: Viral Brahmbhatt, MBBS, PhD, R and D Specialist, Department of Nutrition and Health, Nestlé Research Centre, Vers-chez-les-Blanc 26, PO Box 44, CH-1000 Lausanne, Switzerland. viralvishnuprasad.brahmbhatt@rdls.nestle.com
Telephone: +41-21-7858265 Fax: +41-21-7858544

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Abstract

AIM: To investigate the anti-inflammatory properties of Lacto-Wolfberry (LWB), both *in vitro* and using a mouse model of experimental colitis.

METHODS: The effects of LWB on lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) and interleukin (IL)-6 secretion were assessed in a murine macrophage cell line. *in vitro* assessment also included characterizing the effects of LWB on the activation of NF-E2 related 2 pathway and inhibition of tumor necrosis factor-α (TNF-α)-induced nuclear factor-κB (NF-κB) activation, utilizing reporter cell lines. Following the in vitro assessment, the anti-inflammatory efficacy of an oral intervention with LWB was tested *in vivo* using a preclinical model of intestinal inflammation. Multiple outcomes including body weight, intestinal histology, colonic cytokine levels and anti-oxidative measures were investigated.

RESULTS: LWB reduced the LPS-mediated induction of ROS production [+LPS *vs* 1% LWB + LPS, 1590 ± 188.5 relative luminescence units (RLU) *vs* 389 ± 5.9 RLU, $P < 0.001$]. LWB was more effective than wolfberry alone in reducing LPS-induced IL-6 secretion *in vitro* (wolfberry *vs* 0.5% LWB, 15% ± 7.8% *vs* 64% ± 5%, $P < 0.001$). In addition, LWB increased reporter gene expression *via* the anti-oxidant response element activation (wolfberry *vs* LWB, 73% ± 6.9% *vs* 148% ± 28.3%, $P < 0.001$) and inhibited the TNF-α-induced activation of the NF-κB pathway (milk *vs* LWB, 10% ± 6.7% *vs* 35% ± 3.3%, $P < 0.05$). Furthermore, oral supplementation with LWB resulted in a reduction of macroscopic (-LWB *vs* +LWB, 5.39 ± 0.61 *vs* 3.66 ± 0.59, $P = 0.0445$) and histological scores (-LWB *vs* +LWB, 5.44 ± 0.32 *vs* 3.66 ± 0.59, $P = 0.0087$) in colitic mice. These effects were associated with a significant decrease in levels of inflammatory cytokines such as IL-1β (-LWB *vs* +LWB, 570 ± 245 μg/L *vs* 89 ± 38 μg/L, $P = 0.0106$), keratinocyte-derived chemokine/growth regulated protein-α (-LWB *vs* +LWB, 184 ± 49 μg/L *vs* 75 ± 20 μg/L, $P = 0.0244$), IL-6 (-LWB *vs* +LWB, 318 ± 99 μg/L *vs* 117 ± 18 μg/L, $P = 0.0315$) and other pro-inflammatory proteins such as cyclo-oxygenase-2 (-LWB *vs* +LWB, 0.95 ± 0.12 AU *vs* 0.36 ± 0.11 AU, $P = 0.0036$) and phosphorylated signal transducer and activator of transcription-3 (-LWB *vs* +LWB, 0.51 ± 0.15 AU *vs* 0.1 ± 0.04 AU, $P = 0.057$). Moreover, antioxidant biomarkers, including expression of gene encoding for the glutathione peroxidase, in the colon and the plasma anti-oxidant capacity were significantly increased by supplementation with LWB (-LWB *vs* +LWB, 1.2 ± 0.21 mmol/L *vs* 2.1 ± 0.19 mmol/L, $P = 0.0095$).

CONCLUSION: These results demonstrate the anti-inflammatory properties of LWB and suggest that the underlying mechanism is at least in part due to NF-κB inhibition and improved anti-oxidative capacity.

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Key words: Lacto-Wolfberry; Colitis; Nutrition; Inflammation; Wolfberry; Inflammatory bowel disease; Crohn's disease

Peer reviewers: Dr. Ferenc Sipos, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi 46, 1088 Budapest, Hungary; Dr. Tedros Bezabeh, Institute for Biodiagnostics, National Research Council, 435 Ellice Avenue, Winnipeg R3B 1Y6, Canada; Jae Hee Cheon, Professor, Department of Internal Medicine, Yonsei University College of Medicine, 250 Seongsanro, Seodaemun-gu, Seoul 120-752, South Korea

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INTRODUCTION

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), represents a group of chronic disorders characterized by inflammation of the gastrointestinal tract, typically with a relapsing and remitting clinical course. They affect between 0.5%-1% of the Western world's population[1]. The primary focus of IBD therapy is to induce remission of acute inflammatory flare ups and to maintain the state of remission.

The innate immune system plays a central role in the acute inflammatory process. As part of the innate immune response, neutrophils are one of the early responders to local injury. Both, the circulating levels and activation of neutrophils, are increased in IBD patients with active disease[2,3]. Activated neutrophils and monocytes release a plethora of mediators including reactive oxygen species (ROS), eicosanoids and proinflammatory cytokines. In fact, the therapeutic benefit of depleting granulocytes in CD patients has been demonstrated[4]. Apart from neutrophils, monocytes and mucosal macrophages play an important role in the development of IBD as shown by an increase of the number of recruited monocytes and activated macrophages in the inflamed gut of patients with IBD[5]. Indeed during active inflammation, neutrophils recruit and activate monocytes which themselves secrete pro-inflammatory mediators such as tumor necrosis factor (TNF-α), interleukin (IL)-1β and IL-6[6].

A vast body of literature supports the role of nutritional therapy in IBD, particularly in CD (reviewed in[7,8]). While enteral nutrition is not as effective as steroid therapy in induction of remission in CD, the benefit to the patient is well established[9]. Thus, identification and characterization of novel anti-inflammatory foods may aid in improving the currently available nutritional formulations.

A variety of functional nutrients, such as glycosides[10], alkaloids[11] and black tea extracts[12], have been shown to exert their beneficial effects through inhibition of Nuclear factor-κB (NF-κB) activation. NF-κB is one of the most important regulators of pro-inflammatory cytokine expression and reducing its activity may have beneficial effects under acute inflammatory conditions[13]. Besides NF-κB, phytochemicals are also known to activate NF-E2 related 2 (Nrf2) pathway through the anti-oxidant response element causing an increase in the anti-oxidative enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase[14].

Wolfberry, the fruit of *Lycium barbarum*-also called as Goji (Gouqi or Gou QZ in Romanized Chinese), is a sweet red berry, which has been traditionally used in Chinese medicine. It is also one of the richest sources of zeaxanthin, an antioxidant that has been postulated to improve visual acuity[15]. Apart from antioxidant activity, wolfberry juice has also been demonstrated to have immunomodulatory effects[16]. However, a large part of the supporting evidence is derived either from *in vitro* experiments or animal studies wherein, wolfberry extracts were delivered parenterally. We believe that this might be due to reduced bioavailability of active ingredients when given enterally. Therefore, to improve bioavailability of its anti-oxidant components, wolfberry was processed with skimmed milk and freeze-dried to generate Lacto-Wolfberry (LWB), a water-dispersible powder[17]. This novel preparation, which contains approximately 50% wolfberry and 25% skimmed milk, has been clinically demonstrated to improve the bioavailability of zeaxanthin[17]. Subsequently, the immune-enhancing properties of LWB in both, young-adult and aged mice, have been characterized[18]. Recent studies have demonstrated that dietary supplementation with LWB enhances immune response to flu vaccine[19] and plasma oxidative capacity in elderly[20]. The aim of this study was to characterize the anti-inflammatory and anti-oxidative properties of LWB. We first demonstrate that LWB inhibits lipopolysaccharide (LPS)-induced ROS and IL-6 production in a murine macrophage cell line. Next, using reporter cell lines we show that LWB activates Nrf2 pathway, while inhibiting the NF-κB pathway. Finally, using a mice model of colitis, we demonstrate that LWB reduces the severity of colitis by mediating a reduction in pro-inflammatory cytokines, namely IL-6, IL1β and keratinocyte-derived chemokine/growth regulated protein-α (KC/GRO-α).

MATERIALS AND METHODS

Inflammatory response in LPS-challenged RAW cells

The murine macrophage cell line RAW 264.7 (ATCC, United States) was maintained in Dulbecco's modified Eagle medium (DMEM, Amimed, Bioconcept, Switzerland) supplemented with 10% heat-inactivated fetal-calf serum (FCS, Amimed) at 37 ℃ in a 50 mL/L CO_2/air incubator. Intracellular ROS was measured using a ROS-sensitive fluorescent dye, 2',7'-dichlorofluorescin diacetate (DCFH2-DA, Sigma, United States). Cells (10^5

cells/well in 96 well plates) were incubated overnight with LPS, from *Escherichia coli* serotype 055:B5 (Sigma, United States) at 0.5 mg/L, either in the absence or presence of LWB (0.1% or 1% final concentration). Control cells in the absence of LPS were also included. Cells were then treated with 10 μmol DCFH2-DA for 30 min at 37 ℃ and washed twice with phosphate-buffered saline (PBS). Fluorescence was measured at 485 nm excitation and 538 nm emission by a Fluoroskan enzyme linked immunosorbent assay plate reader (Labsystems Oy, Finland) at the indicated time points. For experiments measuring IL-6, RAW 264.7 cells were seeded in 96 well plates at 10^4 cells/well. After 3 d (approximately 80% of confluence), cells were stimulated with LPS at 0.5 mg/L and incubated in the presence of either LWB (1% final concentration), wolfberry (0.5%) or skimmed milk (0.25%) for 24 h at 37 ℃. Cell culture supernatants were then harvested and IL-6 secretion was quantified using commercial enzyme linked immunosorbant assay (ELISA) kit according to manufacturer's protocol (Murine IL-6 Eli-pair, Diaclone, France). Cell viability was determined by CellTiter-Glow Luminescent assay (Promega, United States) according to manufacturer's instructions. It should be noted that the same lots of wolfberry and skimmed milk that were used in the preparation of LWB were used for all experiments described.

NF-κB inhibition assay

The human colonic adenocarcinoma cell line, HT-29 (ATCC, United States), was stably transfected with the plasmid pNF-κB-SEAP-NPT. The plasmid was a kind gift from Prof. Kim (Natural Products Research Institute, Seoul). It contains a secreted alkaline phosphatase (SEAP) encoding sequence downstream of four tandem copies of NF-κB binding sites. Stably transfected cells (HT-29 clone 34) were maintained in high glucose (4.5 g/L) DMEM containing 1% L-glutamine, 10% heat-inactivated FCS, 1% penicillin/streptomycin, 500 mg/L G418 (Invitrogen, Switzerland) and 100 mg/L Normocin (Invivogen, France) at 37 ℃ in a 50 mL/L CO_2/air incubator. For the NF-κB inhibition assay, HT-29 clone 34 cells were seeded at 10^4 cells/well in 96-flat bottom well plates. After 3-4 d of culture (approximately 80% confluence), cells were washed with PBS and stimulated with recombinant TNF-α (10 μg/L, RD systems, England) in the absence or the presence of either LWB (1% final concentration), wolfberry (0.5%) or skimmed milk (0.25%) for 24 h at 37 ℃. SEAP release was assessed in the supernatants using the Phospha-Light™ System (Applied Biosystems, United States) according to manufacturer's protocol.

Nrf2 activation assay

AREc32 (CXR biosciences, United Kingdom) is a reporter cell line that stably expresses the anti-oxidant response element (ARE)-driven luciferase gene[21]. These cells were cultured at 12 000 cells/well in 96 well plates (Nunc) at 37 ℃ and 50 mL/L CO_2/air incubator in DMEM supplemented with 10% FCS. After 1 d, cells were washed with PBS and treated, in the absence of serum, with either, LWB (1% final concentration), wolfberry (0.5%) or skimmed milk (0.25%) for 24 h at 37 ℃. Luciferase activity was measured using the Luciferase Assay™ (Promega, United States) following manufacturer's instructions.

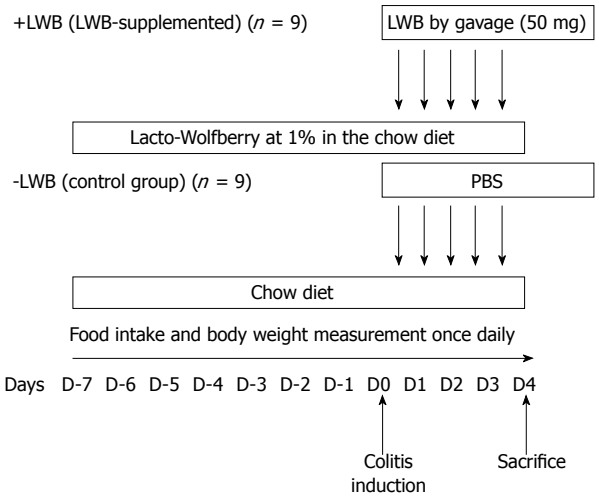


Figure 1 Animal study design. Body weight and food intake was monitored throughout the duration of the experiment (i.e., D-7 to D4). Colitis was induced on D0. For the Lacto-wolfberry (LWB) fed group, diet was supplemented with 1% LWB from D-7 until D4 and 50 mg of LWB was also gavaged from D0 to D4. Control animals were fed regular diet for the same period and gavaged with equal volume of control solution from D0 to D4. PBS: Phosphate buffered solution.

Experimental colitis model

Male mice (C57BL/6J), aged 7 wk, were obtained from Charles River Laboratories Inc. (France) and housed five per cage in a temperature-controlled room with free access to food and water. The overall study design is provided in Figure 1. Mice (n = 9 per group) were randomly assigned to either the control (LWB, chow fed) or the LWB-supplemented group (+LWB, 1% in the diet) 7 d prior to colitis induction (D-7), which was induced on D0, as previously described[22]. Chemically induced colitis was performed as described earlier[23]. Briefly, colitis was induced with an intrarectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) at 125 mg/kg dissolved in 50% ethanol solution. In order to compensate for reduced food intake after colitis induction, mice were also supplemented by daily gavage with 50 mg of LWB from D0 to D4. Control mice were gavaged with an equal volume of PBS. Food intake and body weight measurements were taken once daily from D-7 to D4. Animals were sacrificed on D4. All experimental protocols were conducted in accordance with Swiss law and Nestlé policy on ethics and animal welfare.

Macroscopic and histological assessment

The distal colon tissue was washed with PBS and macroscopic scoring was performed using the system of Wallace *et al*[24]. Samples of the inflamed tissues (1 cm above the anal canal) were collected for histological analysis. The tissues were fixed in 4% paraformaldehyde at 4 ℃

for 24 h. Sections were prepared, stained with hematoxylin and eosin, and graded according to Ameho *et al*[25].

mRNA expression analysis

Colon tissue homogenization, RNA extraction and reverse transcription were performed as described earlier[22]. Custom-made Low Density Array (LDA) cards were purchased from Applied Biosystems (United States) and used according to manufacturer's instructions. Briefly, mixes (100 μL), containing 100 ng of cDNA, 2X TaqMan Mix and nuclease-free water, were prepared and loaded onto the LDA card. The LDA cards were then processed using an automated fluorometer ABI Prism 7900HT. Gene expression was calculated using the relative quantification method with SDS 2.2.2 software.

Electrophoresis and Western blotting analysis

Colon tissue homogenization, protein extraction, electrophoresis and Western blotting analysis were performed as described earlier[22]. Briefly, after tissue homogenization, protein was quantified using the RC DC Protein Assay (Bio-Rad, United States). Proteins were loaded and separated on a 4%-12% bis-tris gel (Invitrogen). The blot was probed with antibodies against murine cyclooxygenase-2 (COX-2) (Cayman, United States), signal transducer and activator of transcription-3 (STAT-3) and phosphorylated STAT-3 (pSTAT-3, Cell Signalling Technology, United States) and β-Actin (Sigma, United States). Relative quantitation of bands was determined using Scion Image Densitometry System (Scion Corp., United States), with normalization to β-Actin.

Myeloperoxidase assay

Protein levels for myeloperoxidase (MPO) were measured in colon protein extracts by ELISA following the manufacturer's instructions (Hycult biotechnology, The Netherlands).

Cytokine analysis

IL-1β, IL-6, IL-10, IL-12p70, KC/GRO-α, interferon-γ (IFN-γ) and TNF-α were measured in the colon protein extracts using multiplex assay kits (Meso Scale Discovery, United States) according to manufacturer's protocol. Cytokine concentrations were determined with Discovery Workbench 3.0 software, using curve 4-PL as suggested by the manufacturer.

Plasma anti-oxidant capacity

Total anti-oxidant capacity of plasma was performed using an assay, which measures inhibition of 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS®) to ABTS®+ by metmyoglobin as Trolox equivalents (Cayman, United States), according to manufacturer's protocol.

Statistical analysis

Data were analyzed by means ± SE either the Mann-Whitney test or where appropriate, a two-way analysis of variance with a Bonferroni post test. *P* values of less than 5% were considered as significant.

RESULTS

Effects of LWB in vitro

Anti-oxidant effects: ROS production from LPS-stimulated RAW 264.7 cells was evaluated in the presence and absence of LWB (Figure 2A). As expected, LPS increased ROS production, in a time dependent manner, as compared to the untreated controls (-LPS). Interestingly, LWB was able to reduce LPS-induced ROS production. The inhibitory effect of LWB was significant from the 2 h time point at both 0.1% and 1% final concentration. At the 6 h time point, LWB reduced the amount of LPS-induced ROS production by about 25% and 75%, at the concentrations of 0.1% and 1%, respectively ($P < 0.001$ for both concentrations). Next, the effects of LWB on Nrf2 activation, using a stable ARE-driven reporter gene expressing cell line, *AREc32*[21], were evaluated (Figure 2B). The data show that LWB at 1% final concentration increased Nrf2 activity by approximately 200%, whereas its individual components, i.e., wolfberry (0.5%) and milk (0.25%), induced only a mild or almost no increase in Nrf2 activity, respectively.

Anti-inflammatory effects: Finally, the effects of LWB (1%), wolfberry (0.5%) and milk (0.25%) on LPS-induced IL-6 production (Figure 2C) and TNF-α-induced NF-κB activity (Figure 2D) were assessed. As shown, LWB inhibited LPS-induced IL-6 production in RAW 264.7 cells by approximately 80% and TNF-α-induced NF-κB activity by approximately 35%. These were significantly different from the values obtained for milk, approximately 20% and 10%, respectively ($P < 0.001$ and $P < 0.05$, respectively). However, no significant differences were observed from wolfberry, which inhibited IL-6 production by approximately 65% and NF-κB activation by 10%.

Anti-inflammatory effects of LWB in vivo

The anti-inflammatory effects of LWB were characterized in chemically-induced colitis model as described in materials and methods section.

LWB attenuates colitis-induced body weight loss: Body weight and food intake of each mouse was monitored daily. Following colitis induction at D0, the percentage mean change in body weight of control mice was -6.4, -9.5, -8.9 and -7.6 at D1, D2, D3 and D4, respectively (Figure 3, LWB). The mice fed with LWB had a percentage mean change in body weight of -4.7, -4.6, -1.9 and -0.5 at D1, D2, D3 and D4, respectively (Figure 3, +LWB). Thus, while both mice have a reduction in body weight at D1, the reduction in body weight loss of the LWB fed mice were significantly lower from D2-D4 ($P < 0.05$ at D2 and $P < 0.01$ at D3 and D4). The total food intake between the two groups did not change (data not shown).

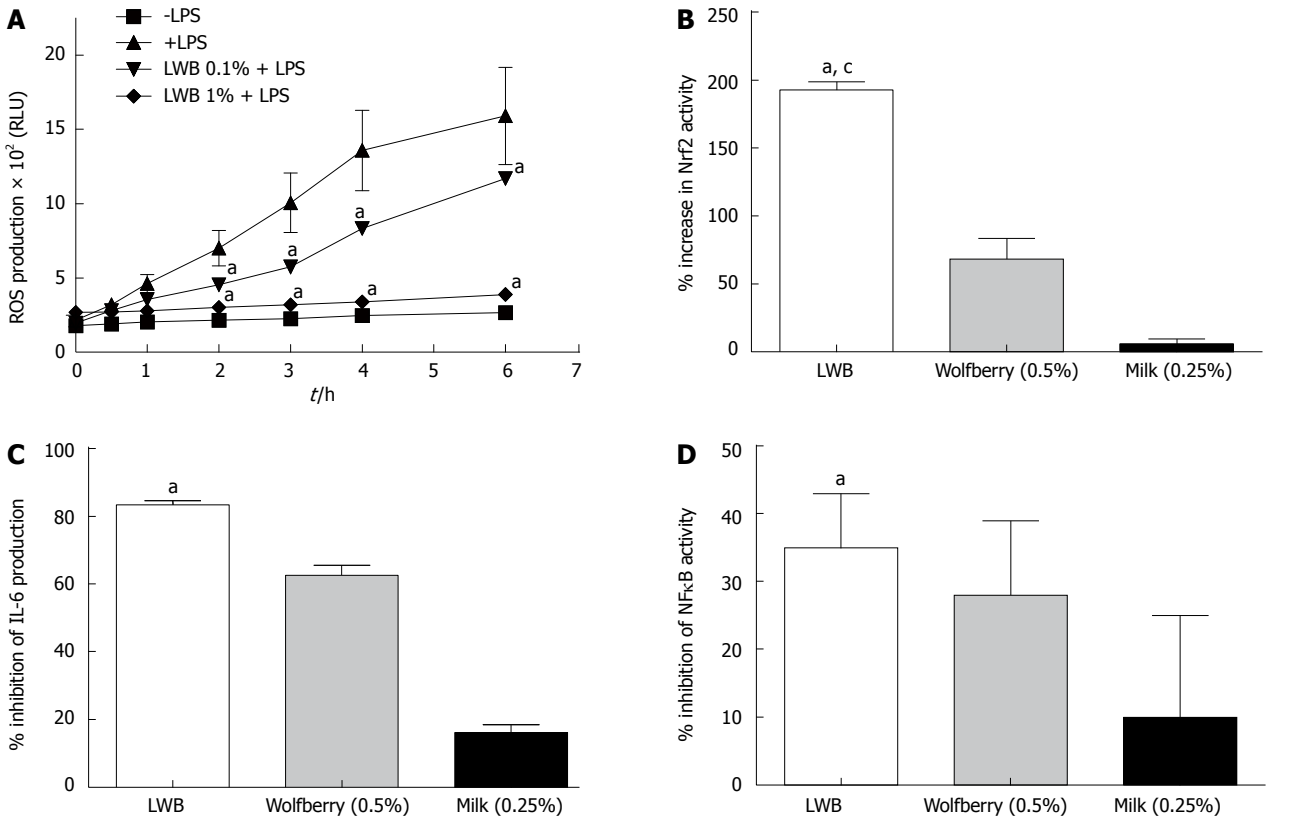


Figure 2 Anti-inflammatory and anti-oxidative effects of Lacto-Wolfberry *in vitro*. A: The effect of Lacto-Wolfberry (LWB) on lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) production in RAW 264.7 cells was measured as described in the methods section. Results are expressed in relative luminescence units (RLU). $^{a}P < 0.05$ at the given time points and dose as compared to LPS-stimulated ROS production (+LPS); B: Effects of given concentrations of LWB, milk and wolfberry on NF-E2 related 2 activity in AREc32 cells. Results are expressed as percentage of increase compared to control conditions. $^{a}P < 0.05$ *vs* milk; $^{c}P < 0.05$ *vs* wolfberry; C: Effects of given concentrations of LWB, milk and wolfberry on LPS-induced interleukin-6 production in RAW 264.7 cells. Results are expressed as percentage of inhibition compared to induction after LPS stimulation. $^{a}P < 0.05$ *vs* milk; D: Effects of given concentrations of LWB, milk and wolfberry on tumor necrosis factor-α-induced nuclear factor- κB activation in a reporter cell line. Results are expressed as percentage of inhibition compared to induction after TNF-α stimulation. All results are depicted as mean ± SE. $^{a}P < 0.05$ *vs* milk.

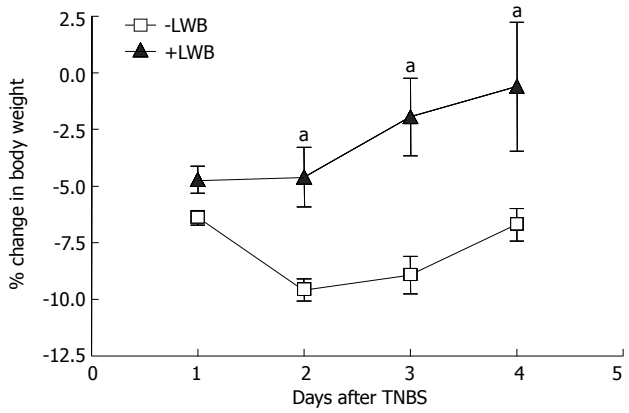


Figure 3 Lacto-Wolfberry attenuates colitis-induced body weight loss. Percentage change in body weight was calculated from a ratio of body weight measured at each day after colitis induction and body weight at D0. Results are depicted as mean ± SE. $^{a}P < 0.05$ *vs* -Lacto-Wolfberry (LWB) at the respective time points. TNBS: 2,4,6-trinitrobenzene sulfonic acid.

LWB reduces colonic inflammation: Supplementation with LWB significantly reduced the colonic inflammation as judged by macroscopic (Figure 4A) and histological (Figure 4B, C and D) evaluation of intestinal inflammation. Macroscopic lesions were assessed as delineated by Wallace *et al*[24]. Colons of control mice (-LWB) presented significantly higher scores compared to mice fed with LWB, 5.39 ± 0.61 and 3.66 ± 0.47, respectively (Figure 4A, $P < 0.05$). In agreement with the macroscopic assessment, histological evaluation showed lower inflammatory infiltrates and better mucosal integrity in mice fed with LWB (+LWB, Figure 4D) as compared to the control (-LWB, Figure 4C). This difference was reflected in the histological score, 5.44 ± 0.32 and 3.66 ± 0.59, for the control and LWB-fed mice, respectively, which was significantly different (Figure 4B, $P < 0.01$). The 70% reduction of MPO content in the LWB treated group (+LWB) as compared to control mice (-LWB) provides further support to reduced neutrophil infiltration in the LWB group (Figure 4E, $P < 0.05$).

LWB reduces pro-inflammatory effector proteins: To delve deeper into the effect of LWB, levels of various effector proteins were measured in the colon tissue. As shown in Table 1, levels of IL-1β, IL-6 and KC/GRO-α were significantly reduced in colitic mice fed with LWB (+LWB) compared to the control (-LWB). Moreover IL-10, IFN-γ, TNF-α and IL-12p70 levels were also re-

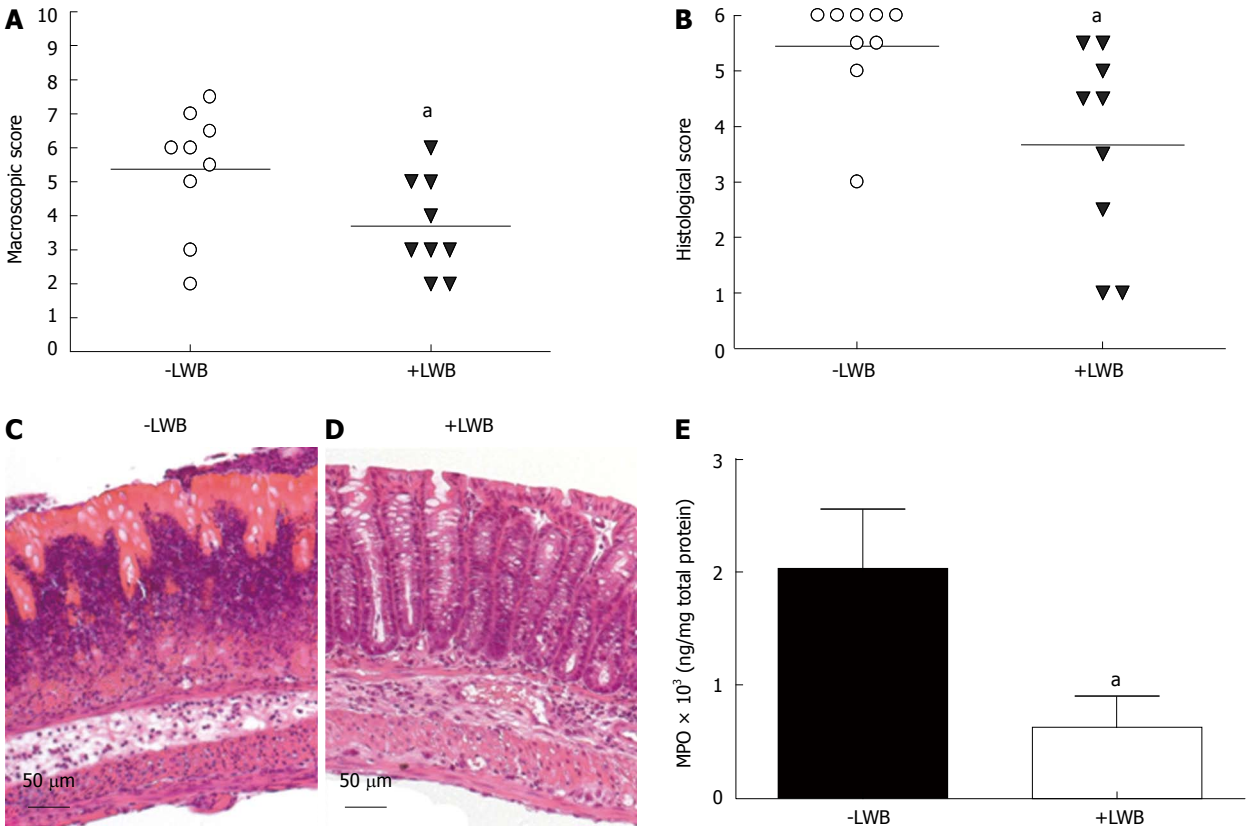


Figure 4 Lacto-Wolfberry reduces colonic inflammation. A: Macroscopic evaluation of the colon was performed according to Wallace criteria. Results are expressed in individual values and the mean value is represented by the horizontal black line; B: Histological evaluation and scoring of the colon was performed according to the Ameho criteria. The individual scores of the mice in the two groups are provided, with the mean value being represented by the horizontal line; C: Representative section of score 6 from the control fed mice [-Lacto-Wolfberry (LWB)]; D: Representative section of score 1 from LWB fed mice (+LWB); E: Colonic myeloperoxidase (MPO) levels were measured by enzyme linked immunosorbant assay. The values are represented as mean ± SE. $^{a}P < 0.05$ *vs* -LWB.

Table 1 Cytokine levels in colon samples (mean ± SE)

Cytokines (μg/L)	-LWB	+LWB	Reduction (%)	*P* value
TNF-α	80 ± 24	31 ± 4	61	0.0770
IL-1β	570 ± 245	89 ± 38	84	0.0106
IL-6	318 ± 99	117 ± 18	63	0.0315
KC/GRO-α	184 ± 49	75 ± 20	59	0.0244
IL-12p70	415 ± 129	89 ± 38	79	0.1135
IFN-γ	18 ± 6	7 ± 1	61	0.0770
IL-10	428 ± 141	165 ± 27	61	0.0625

LWB: Lacto-Wolfberry; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β; IL-6: Interleukin-6; KC/GRO-α: Keratinocyte-derived chemokine/growth regulated protein-α; IL-12p70: Interleukin-12p70; IFN-γ: Interferon-γ; IL-10: Interleukin-10.

duced by more than 50%, however these were not statistically significant. COX-2 and pSTAT3 levels were assessed semi- quantitatively by Western blotting analysis and densitometry. As shown in Figure 5A, colon of mice fed with LWB had approximately 65% reduction of COX-2 levels ($P < 0.01$). Mice fed with LWB also demonstrated an 80% reduction in pSTAT3 expression (Figure 5B) in the colon. However, this difference didn't reach statistical significance ($P = 0.057$).

LWB improves anti-oxidative capacity: Finally, changes in mRNA expression of Nrf2 target genes, such as CAT, SOD2 and glutathione peroxidase (GPx1) were examined in the colon tissue. GPx1 mRNA expression was higher by more than 20% in mice fed with LWB (+LWB) compared to control (-LWB) (Figure 6A, $P < 0.02$). No difference of gene expression was detected for CAT and SOD2 (data not shown). Finally, the measures of anti-oxidant capacity in the plasma demonstrated that mice supplemented with LWB (+LWB) had more than 70% increase in anti-oxidative capacity compared to control mice (-LWB) (Figure 6B, $P < 0.01$).

DISCUSSION

The aim of the study was to investigate the anti-inflammatory properties of LWB *in vitro* and in an animal model of intestinal inflammation. Initial experiments showed that LWB reduces LPS-induced ROS generation. The anti-oxidant effects of wolfberry are well characterized[16]. Moreover, LWB has also been proposed to have ROS scavenging activity[26]. Thus, this finding was not surprising. However, phytochemicals have also been shown to activate the Nrf2 pathway[14]. Nrf2 is a redox-sensitive transcription factor, which regulates the expression of ARE-driven anti-oxidant enzymes[14]. Thus, the effect of LWB on Nrf2 activation was tested. Interestingly, LWB

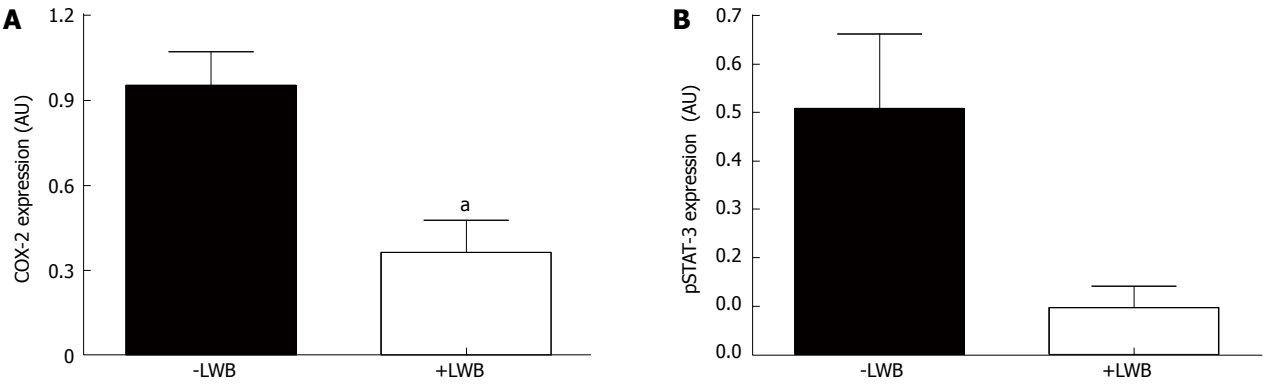


Figure 5 Effect of Lacto-Wolfberry on cyclooxygenase-2 and phosphorylated- signal transducer and activator of transcription-3 levels. A: Cyclooxygenase-2 (COX-2) levels were assessed semi-quantitatively by Western blotting and densitometry. COX-2 levels were normalized to β-actin. Results are expressed in mean ± SE. [a]*P* < 0.05 *vs* -Lacto-Wolfberry (LWB); B: Phosphorylated-signal transducer and activator of transcription-3 (pSTAT3) levels were assessed by Western blotting and densitometry. The results are expressed as a ratio of pSTAT3 to STAT3 (mean ± SE). *P* = 0.057 *vs* -LWB group.

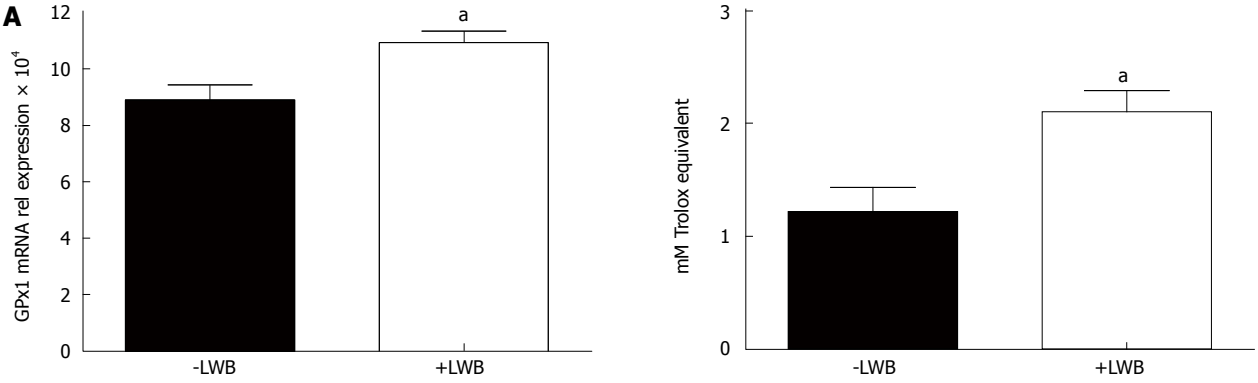


Figure 6 Anti-oxidant effect of Lacto-Wolfberry in murine colitis. A: mRNA expression of glutathione peroxidase 1 (GPx1) was measured as described in methods section. Results are expressed as mean ± SE; B: Anti-oxidant ability in plasma was measured in mmol/L Trolox equivalent. Results are expressed in mean ± SE. [a]*P* < 0.05 *vs* -Lacto-Wolfberry (LWB).

had a two fold increase in Nrf2 activation, however this effect was not fully replicated by its major components tested separately, i.e., either, wolfberry or milk. Hence, this could suggest a synergistic effect between the two major components of LWB resulting from the LWB manufacturing process. Finally, LWB demonstrated wolfberry-equivalent inhibition of LPS-induced IL-6 secretion and TNF-α-induced NF-κB activation. The beneficial effects observed *in vitro* prompted a further examination of LWB in an animal model of colitis.

The pro-inflammatory roles of ROS production, NF-κB activation and cytokines, such as IL-1β, IL-6, IL-8 and TNF-α, have been firmly established in IBD pathology[13,27,28]. Thus, the effects of LWB were tested in a murine model of colitis. Firstly, LWB supplementation attenuated colitis-induced body weight loss. Secondly, both colonic parameters, macroscopic and microscopic, confirmed a reduction in the severity of colitis after LWB intervention. In further support, supplementation with LWB reduced neutrophil infiltration in the colon tissue. In this experimental model, the secretion of Th1 cytokines, namely, IL-1β, IL-6, TNF-α and IFN-γ, play an important role in the propagation of colitis[29]. As NFκB activation controls expression of most of these genes[13] and considering the *in vitro* inhibitory effects of LWB on NF-κB activation, the cytokine levels were measured to gain mechanistic insight. Supplementation with LWB resulted in reduced levels of majority of these cytokines, while significantly reducing the levels of IL-1β and IL-6. Further, LWB supplementation reduced not just the levels of IL-6, but also the downstream signaling *via* STAT-3 activation. Interestingly however, there was only non significant trend observed in the reduction of TNF-α. It is possible that this could be either due to technical variability or due to different kinetics of this cytokine. Moreover, the significant reduction in KC/GRO-α, an established chemokine involved in neutrophil chemotaxis[30], can explain the reduction in neutrophil infiltration. Thus, modulation of cytokine levels, perhaps *via* a decrease in NF-κB activation, is responsible for the anti-inflammatory effects of LWB in this study. The increase of Nrf2 activation *in vitro* as well as an increase in the plasma anti-oxidative capacity and upregulation of GPx1 after LWB supplementation in colitic mice also suggests a possibility of an anti-oxidant mechanism underlying the anti-inflammatory effects of LWB. However, the lack of upregulation of other Nrf2 target genes, such as, CAT and SOD2 means that the anti-oxidative properties explains only part of

the overall effects observed.

Wolfberry is believed to contain at least three different biologically active components: (1) Lycium Barbarum polysaccharides (LBP); (2) zeaxanthin dipalmitate; and (3) 2-O-β-D-glucopyranosyl-L-ascorbic acid (a Vitamin C analogue)[16]. The anti-oxidative properties of all three active ingredients of wolfberry are well documented[16]. LWB is prepared by a milk based extraction process of wolfberry, which is believed to increase bioavailability of its active ingredients, as demonstrated for zeaxanthin[17]. In addition, the *in vitro* assays suggest that the anti-inflammatory activity of LWB is attributable to wolfberry rather than its milk component. However, to the best of our knowledge, wolfberry has not been shown to reduce cytokine levels under inflammatory conditions, as observed in our present study. In fact, wolfberry has been shown to up-regulate cytokine expression[31] and both LWB and wolfberry have a demonstrated immune-enhancing effect[18,19,32-34]. On the other hand, the anti-inflammatory properties of milk components are well established[35,36]. Overall, it seems that depending on the physiological environment LWB may provide support to recover homeostasis and/or immune competence. In the present study, we can speculate that a synergistic effect between the anti-oxidative ingredients of wolfberry and the anti-inflammatory components in milk can also be a potential mechanism of the benefits observed in our model. In that respect, further studies are required to identify the active anti-inflammatory ingredients in LWB. Nutritional therapies are an effective and safe form of intervention to induce remission in active state of CD[7,8]. Despite this profile of effectiveness and safety, they have not gained widespread usage, particularly in the area of adult IBD. One of the reasons for this could be that while they are effective, a review of the clinical trials comparing the efficacy of nutritional therapies to steroids concluded that, they are not as effective as steroids in induction of remission[9]. Thus, clearly more needs to be done in this area. Our findings suggest that addition of LWB to enteral diet formulations might help improve disease outcomes in IBD patients. However, it should be noted that further work addressing efficacy in different colitis models and in-depth confirmation of mechanism of action is necessary before clinically relevant research can be undertaken.

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COMMENTS

Background

Inflammatory bowel disease (IBD) consists of a group of disorders, such as Crohn's disease (CD) and ulcerative colitis (UC). The incidence of IBD is increasing throughout the world. Both, CD and UC are characterized by relapsing-remitting disease progression. Currently, there is no known cure for IBD and the various available therapies are only palliative. Herein, the authors have identified the anti-inflammatory properties of a nutritional ingredient in a preclinical model of colitis.

Research frontiers

Due to the chronic nature of IBD and the adverse effects of existing therapies, nutritional ingredients with anti-inflammatory properties may benefit the patient in the long term. With this in mind, the characterization of anti-inflammatory properties of novel or traditional food ingredients is an important field of research.

Innovations and breakthroughs

Previously, the authors have characterized the benefits of Lacto-Wolfberry (LWB) on the adaptive immune system. In this article, they have characterized the anti-inflammatory properties of LWB. The authors first demonstrate the anti-inflammatory and anti-oxidative properties of LWB in cellular models and subsequently show that LWB can ameliorate chemically-induced colitis.

Applications

The identification of the anti-inflammatory properties of LWB raises new possibilities of developing novel nutritional solutions for patients with IBD.

Terminology

LWB is a skimmed milk extract of the traditional Chinese ingredient, wolfberry, specifically developed to increase the bioavailability of its active ingredients.

Peer review

In the original article, the authors examined the complex anti-inflammatory effect of LWB administration in 2,4,6-trinitrobenzene sulfonic acid induced colitis and in selected cell lines. The study is well designed and the results and conclusions are clear and logical.

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ORIGINAL ARTICLE

Increased tumor necrosis factor receptor 1 expression in human colorectal adenomas

Kunihiro Hosono, Eiji Yamada, Hiroki Endo, Hirokazu Takahashi, Masahiko Inamori, Yoshitaka Hippo, Hitoshi Nakagama, Atsushi Nakajima

Kunihiro Hosono, Eiji Yamada, Hiroki Endo, Hirokazu Takahashi, Masahiko Inamori, Atsushi Nakajima, Division of Gastroenterology, Yokohama City University School of Medicine, Yokohama 2360004, Japan
Yoshitaka Hippo, Hitoshi Nakagama, Division of Cancer Development System, National Cancer Center Research Institute, Tokyo 1040045, Japan
Author contributions: Hosono K performed most of the research and wrote the paper; Yamada E, Endo H and Takahashi H performed the endoscopies; Inamori M analyzed the data; Hippo Y, Nakagama H and Nakajima A were major contributors in revising the manuscript critically for important intellectual content.
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Correspondence to: Dr. Atsushi Nakajima, Professor, Division of Gastroenterology, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 2360004, Japan. nakajima-tky@umin.ac.jp
Telephone: +81-45-7872640 Fax: +81-45-7843546
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Abstract

AIM: To determine the expression statuses of tumor necrosis factor (TNF)-α, its receptors (TNF-R) and downstream effector molecules in human colorectal adenomas.

METHODS: We measured the serum concentrations of TNF-α and its receptors in 62 colorectal adenoma patients and 34 healthy controls. The protein expression of TNF-α, TNF-R1, TNF-R2 and downstream signals of the TNF receptors, such as c-Jun N-terminal kinase (JNK), nuclear factor-κ B and caspase-3, were also investigated in human colorectal adenomas and in normal colorectal mucosal tissues by immunohistochemistry. Immunofluorescence confocal microscopy was used to investigate the consistency of expression of TNF-R1 and phospho-JNK (p-JNK).

RESULTS: The serum levels of soluble TNF-R1 (sTNF-R1) in adenoma patients were significantly higher than in the control group (3.67 ± 0.86 ng/mL *vs* 1.57 ± 0.72 ng/mL, $P < 0.001$). Receiver operating characteristic analysis revealed the high diagnostic sensitivity of TNF-R1 measurements (AUC was 0.928) for the diagnosis of adenoma, and the best cut-off level of TNF-R1 was 2.08 ng/mL, with a sensitivity of 93.4% and a specificity of 82.4%. There were no significant differences in the serum levels of TNF-α or sTNF-R2 between the two groups. Immunohistochemistry showed high levels of TNF-R1 and p-JNK expression in the epithelial cells of adenomas. Furthermore, a high incidence of co-localization of TNF-R1 and p-JNK was identified in adenoma tissue.

CONCLUSION: TNF-R1 may be a promising biomarker of colorectal adenoma, and it may also play an important role in the very early stages of colorectal carcinogenesis.

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Key words: Tumor necrosis factor-α; Tumor necrosis factor receptor 1; c-Jun N-terminal kinase; Colorectal adenoma; Biomarker

Peer reviewers: Peter Laszlo Lakatos, MD, PhD, 1st Department of Medicine, Semmelweis University, Koranyi S2A, H1083 Budapest, Hungary; Annika Lindblom, Professor, Molecular Medicine and Surgery, Karolinska Institutet, S17176 Stockholm, Sweden; Manuel Romero-Gomez, Professor, Department of Medicine, University of Sevilla, Avda. de Bellavista, 41014 Sevilla, Spain

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INTRODUCTION

The proinflammatory cytokine, tumor necrosis factor (TNF), plays an important role in diverse cellular events, such as the induction of other cytokines' expression, cell proliferation, differentiation, necrosis and apoptosis[1,2]. Many of the TNF-induced cellular responses are mediated by one of two specific cellular membrane receptors, tumor necrosis factor receptor 1 (TNF-R1) and TNF-R2, both of which belong to the TNF receptor superfamily[3]. In response to TNF treatment, activation of nuclear factor-κ B (NF-κB), a transcription factor, as well as mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase, p38, and c-Jun N-terminal kinase (JNK) have been reported in most cell types and, in some cases, after apoptosis or necrosis had been induced[4,5].

Overexpression of TNF-α and its receptors often accompanies tumor development and progression, and their important role in the pathogenesis of cancer is now increasingly recognized. Several studies have reported on the status of TNF-α expression in various malignancies. For example, TNF-α mRNA and protein have been detected in both malignant and stromal cells in human ovarian cancer biopsies[6,7]. The plasma levels of TNF-α have been reported to be increased in some cancer patients, especially those with poor prognoses[8-11]. Blood TNF-α concentrations have been reported to be higher in prostate cancer patients with advanced, cachectic disease[12]. These findings suggest that TNF-α might also have an important role in carcinogenesis. We now know that many of the cells and mediators of inflammation that have been detected in human and experimental cancers and inflammatory conditions increase the risk of cancer development[13-16]. There is strong evidence to suggest that cancer-related inflammation aids in the proliferation and survival of malignant cells, stimulates angiogenesis and metastasis[17-19], subverts adaptive immunity[20-22], and modulates their responses to hormones and chemotherapy[23,24].

In colitis-related colon cancer, TNF-α has been demonstrated to promote carcinogenesis, and anti-TNF-α drugs are being used therapeutically[25,26]. Moreover, serum TNF-α is known to serve as an important pathophysiologic marker for the presence and severity of inflammatory bowel disease[27]. However, TNF-α has a short half-life (20 min); therefore, accurate measurement of the blood levels is difficult[28]. There is some debate about whether TNF-α can actually be measured using some sort of physiologically active substance[28,29]. On the other hand, TNF-R1 and TNF-R2 are released into the blood as soluble TNF-R1 (sTNF-R1) and soluble TNF-R2 (sTNF-R2) after proteolysis of their extracellular domains in response to activators, including TNF-α itself[30,31]. Their presence in the peripheral blood is reflective of an inflammatory response occurring within the body. sTNF-R1 and sTNF-R2 also have very long half-lives; thus, they are more stable than TNF-α[32,33]. These receptors may provide better serum biomarkers than TNF-α. Spoettl *et al*[34] found that serum sTNF-R1 levels were significantly increased in ulcerative colitis (UC) patients compared with that of healthy controls. However, there have been few reports of the association between TNF-R1 and TNF-R2 and sporadically occurring colorectal neoplasms[35]. Thus, the association of these receptors with the risk of colorectal adenoma has not yet been fully clarified. The aim of this study was to investigate the serum levels of TNF-α, sTNF-R1 and sTNF-R2 in adenoma patients and also to investigate the expressions of downstream molecules in the TNF-α signaling pathway, including TNF-R1, TNF-R2, JNK, NF-κB and caspase-3 in adenoma tissues in comparison to those in normal colorectal mucosa.

MATERIALS AND METHODS

Patients

The study population consisted of 62 consecutive patients with colorectal adenoma who underwent colonoscopy from January 2008 to January 2009 at the Division of Gastroenterology, Yokohama City University School of Medicine. Patients with concomitant diseases, including infectious diseases, inflammatory bowel diseases, autoimmune conditions, allergy or asthma, that were likely to elevate serum TNF-α levels were excluded from the study. The patients ranged in age from 54 to 86 years (mean ± SD: 67.7 ± 8.2 years) and consisted of 39 males and 23 females. The control group consisted of 34 healthy people, matched for age. The control group consisted of people undergoing colonoscopy after positive fecal occult blood test but not found to have adenomas. The study was carried out in accordance with the Declaration of Helsinki (revised 1989) and with the approval of the Ethics Committee of Yokohama City University School of Medicine. Informed consent was obtained from each patient participating in this study and for the use of the obtained data for research purposes.

Serum samples

Before colonoscopy, blood samples were obtained from all participants after an overnight fast to determine the serum concentrations of TNF-α, sTNF-R1 and sTNF-R2. The blood samples were centrifuged at 800 g for 5 min. Serum was separated as soon as possible from the clot of red blood cells by centrifugation to avoid TNF-α production by the blood cells, which would have led to falsely increased values[36]. Then, the serum specimens were stored at -80 ℃ until the biochemical analyses.

Biochemical determinations

Serum concentrations of human TNF-α, sTNF-R1 and sTNF-R2 were determined by enzyme-linked immunosorbent assay (ELISA) kit (R and D Systems Inc., Minneapolis, MN, United States). The minimum limits of detection of the assay were as follows: TNF-α, 1.6 pg/mL; sTNF-R1, 0.77 pg/mL; and sTNF-R2, 0.6 pg/mL. The tests were carried out as described in the product manuals.

Immunohistochemical analysis

Immunohistochemical analysis was performed in the adenomatous polyps retrieved from the study patients (n = 62). Adenomatous polyps removed during colonoscopy, together with normal colorectal mucosal biopsies from the same patients when possible, were examined. Four-micrometer sections were prepared from the formalin-fixed, paraffin-embedded tissues and mounted on slides coated with polylysine. We examined only sporadically occurring polyps by excluding specimens from patients with familial adenomatous polyposis (FAP) or hereditary non-polyposis coli (HNPCC). The protein expression levels were determined in all 62 normal and colorectal adenomatous tissue sections by immunohistochemistry. Sections were deparaffinized in xylene and rehydrated. The sections were then heated in a 750 W microwave three times for 7 min. Inhibition of endogenous peroxidase activity was performed by treating sections with 3% hydrogen peroxide for 10 min. After washing three times with Tris-Buffered Saline (TBS), the blocking of non-specific interactions was accomplished by incubating the samples with blocking serum for 30 min at room temperature. Sections were then probed with the primary antibodies (Table 1) and subsequently incubated with the Histofine simple stain max PO kit for 30 min (Nichirei Laboratories, Tokyo, Japan) in accordance with the manufacturer's instructions. The signals were visualized by treatment with diaminobenzidine (peroxidase substrate kit, Vector Laboratories, Burlingame, CA, United States), and the sections were counterstained with hematoxylin. Six random microscopic fields per sample of approximately 250 cells were counted at a magnification of 400 × under a bright-field microscope. The results were expressed as the percentage of positive cells with separating epithelial cells and stromal cells.

Immunofluorescence confocal microscopy

For immunofluorescence studies, the sections were stained with anti-TNF-R1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States) at a dilution of 1:50, anti-TNF-R2 rabbit polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:50, and anti-p-JNK mouse monoclonal antibody at Thr 183 and Tyr 185 (Santa Cruz Biotechnology) at a dilution 1:50. After thorough washes in TBS, sections were incubated with their respective Alexa594-conjugated anti-rabbit IgG secondary antibodies (Invitrogen, Carlsbad, CA, United States) or Alexa488-conjugated anti-mouse IgG secondary antibodies (Invitrogen) for 1 h, washed, and mounted.

The confocal imaging was carried out on a FV1000-D (Olympus, Tokyo, Japan) confocal laser scanning microscope. Excitation and detection of the samples were carried out in sequential modes to avoid overlapping of signals. Sections were scanned simultaneously at both wavelengths (488/594 nm) with appropriate laser intensity, confocal aperture, and gain. The Black-level setting was kept constant for all samples.

Table 1 Summary of the specific antibodies used and immunohistochemistry

Protein	Antibody (catalog number)	Dilution	Incubation
TNF-α	Rabbit polyclonal (Abcam, ab6671)	1:100	Overnight
TNF-R1	Rabbit polyclonal (Abcam, ab19139)	1:2000	1 h
TNF-R2	Rabbit polyclonal (Abcam, ab15563)	1:50	30 min
p-JNK	Rabbit polyclonal (CST, #9251)	1:100	Overnight
NF-κB	Rabbit polyclonal (CST, #3034)	1:50	Overnight
Caspase-3	Rabbit polyclonal (CST, #9661)	1:200	Overnight

Abcam (Cambridge, United Kingdom). CST: Cell signaling technology (Beverly, MA, United States).

Table 2 Comparison of the clinical data and measured biochemical parameters in the serum of patients with adenomas and control subjects (mean ± SD)

Parameters	Control (n = 34)	Adenoma (n = 62)	P value
Age (yr)	67.6 ± 15.9	67.7 ± 8.2	NS
Sex (F/M)	14/20	23/39	NS
BMI	23.5 ± 5.0	23.9 ± 3.4	NS
TNF-α (pg/mL)	5.56 ± 1.3	4.83 ± 1.9	NS
sTNF-R1 (ng/mL)	1.57 ± 0.72	3.67 ± 0.86	< 0.001
sTNF-R2 (ng/mL)	2.72 ± 1.15	3.09 ± 0.91	NS

P < 0.05 was denoted significance. NS: Non-significant; TNF-α: Tumor necrosis factor-α; sTNF-R1/2: Soluble tumor necrosis factor receptor 1/2; F/M: Female/male; BMI: Body mass index.

Statistical analysis

Data were expressed as the mean ± SD unless otherwise indicated. The significances of the differences in clinical characteristics between patients with adenomas and controls were evaluated by the χ^2-test for categorical variables and Welch's test for continuous variables. One-way analysis of variance was performed to compare the prevalence of each variable between groups. To assess the diagnostic sensitivity and specificity of the sTNF-R1 and sTNF-R2 measurements, receiver operating characteristic (ROC) curve analysis was performed. P < 0.05 was considered to indicate statistical significance.

RESULTS

Biochemical determinations

Table 2 shows a comparison of the clinical data and laboratory parameters between the 62 patients with adenoma and the 34 normal controls. No differences in

Table 3 Association between the measured biochemical parameters and the number of colorectal adenomas per person

	Control (n = 34)	≤ 3 (n = 34)	≥ 4 (n = 28)	P value
TNF-α (pg/mL)	5.63 ± 1.3	4.31 ± 1.9	4.95 ± 2.0	NS
sTNF-R1 (ng/mL)	1.57 ± 0.72	3.37 ± 0.93	4.05 ± 0.55	< 0.001
sTNF-R2 (ng/mL)	2.72 ± 1.15	3.13 ± 0.88	3.04 ± 0.95	NS

Values are expressed as the mean ± SD; $P < 0.05$ denoted significance. NS: Non-significant; TNF-α: Tumor necrosis factor-α; sTNF-R1/2: Soluble tumor necrosis factor receptor 1/2.

the age, sex or body mass index were found between the two studied groups. The mean serum levels of sTNF-R1 were significantly higher in the adenoma patients compared to the control group patients. On the other hand, there were no significant differences in the serum levels of TNF-α or sTNF-R2 between the two groups. Linear contrast analysis was conducted to evaluate the correlation between each variable and the number of adenomas per person (Table 3). The serum levels of sTNF-R1 were positively correlated with the number of adenomas in the colorectum ($P < 0.001$). As assessed by nonparametric analysis of the ROC curves, sTNF-R1 measurements showed a high diagnostic sensitivity at a value of 0.928 (Figure 1). The best cut-off level for TNF-R1 as 2.08 ng/mL, which showed a sensitivity of 93.4% and a specificity of 82.4%.

Immunohistochemical analysis

Immunohistochemistry showed varying expression intensities of TNF-α, TNF-R1, TNF-R2, p-JNK, NF-κB and caspase-3 in different areas of the samples. The expression of TNF-α in the stromal area of adenomas was significantly higher than that in the stromal areas of the normal mucosa ($P < 0.05$); however, the expression of TNF-α in the epithelial cells of adenomas was not as high as that in the epithelial cells of the normal mucosa (Figure 2). The expression of TNF-R1 in the epithelial cells of the adenomas was significantly higher than that in the epithelial cells of the normal mucosa ($P < 0.05$); however, in the stromal areas, the expression of TNF-R1 was equivalent between the adenomas and the normal mucosa. No significant difference in the expression of TNF-R2 was noted in either the epithelial cells or the stromal areas between the adenomas and the normal mucosa. In relation to the expressions of the downstream molecules in the TNF-α signaling pathway, the expression of p-JNK in both the epithelial cells and stromal areas of the adenoma were significantly higher than those in the normal mucosa ($P < 0.05$) (Figure 3). However, there were no significant differences in the expressions of NF-κB or caspase-3 in either the epithelial cells or stromal areas between the adenomas and the normal mucosa.

To investigate the consistency of TNF-R1 and p-JNK expression, we performed immunofluorescence staining of the adenoma tissues, because the results of immunohistochemistry revealed high levels of TNF-R1 and p-JNK expression in the epithelial cells of the adenomas. The results revealed expression of TNF-R1 and p-JNK co-localized predominantly in the epithelial cells of the adenomas. Merged images of TNF-R1 and p-JNK immunofluorescence staining demonstrated this co-localization (Figure 4). On the other hand, the expression of TNF-R2 was localized predominantly to the stromal area of the adenomas, and merged images of TNF-R2 and p-JNK immunofluorescence staining showed a lack of co-localization.

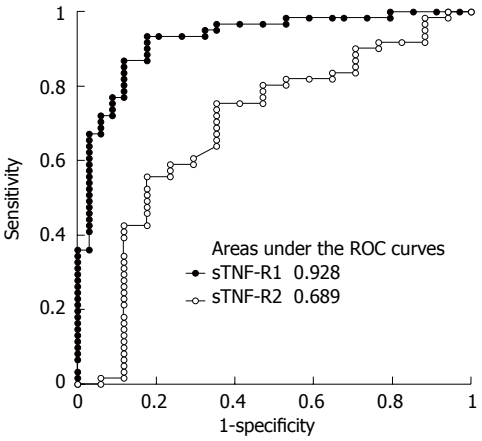


Figure 1 Receiver operating characteristic curves for soluble tumor necrosis factor receptor-1 and 2 in patients with adenomas. ROC: Receiver operating characteristic.

DISCUSSION

This is the first study to show marked changes in the expression levels of TNF-R1 in colorectal adenoma tissues. The serum sTNF-R1 levels were also significantly higher in colorectal adenoma patients than in the control subjects. To evaluate the possible usefulness of TNF-R1 as a biomarker for colorectal adenoma, we evaluated the correlation between the number of adenomas in the colorectum and the serum sTNF-R1 level. The results showed that the serum sTNF-R1 level positively correlated with the number of colorectal adenomas. A ROC analysis revealed the high diagnostic sensitivity and specificity of sTNF-R1 measurements for diagnosis of colorectal adenoma. These results may suggest that TNF-R1 is a promising biomarker for colorectal adenomas. In addition, our results agreed with the Kaminska *et al*[37] study, which showed that sTNF-R1 had the highest diagnostic sensitivity in colorectal cancer patients. Other previous reports have shown a high diagnostic and prognostic accuracy of serum sTNF-R1 levels for various diseases. Determination of the TNF-R1 levels in several body fluids, including the serum, provides valuable insight into a variety of pathological conditions. For example, in cervical adenocarcinoma patients, serum sTNF-R1 is reported to be a useful marker, especially in the early stages of disease[38]. In patients with breast cancer, serum sTNF-R1 is considered to be an independent and clinically useful

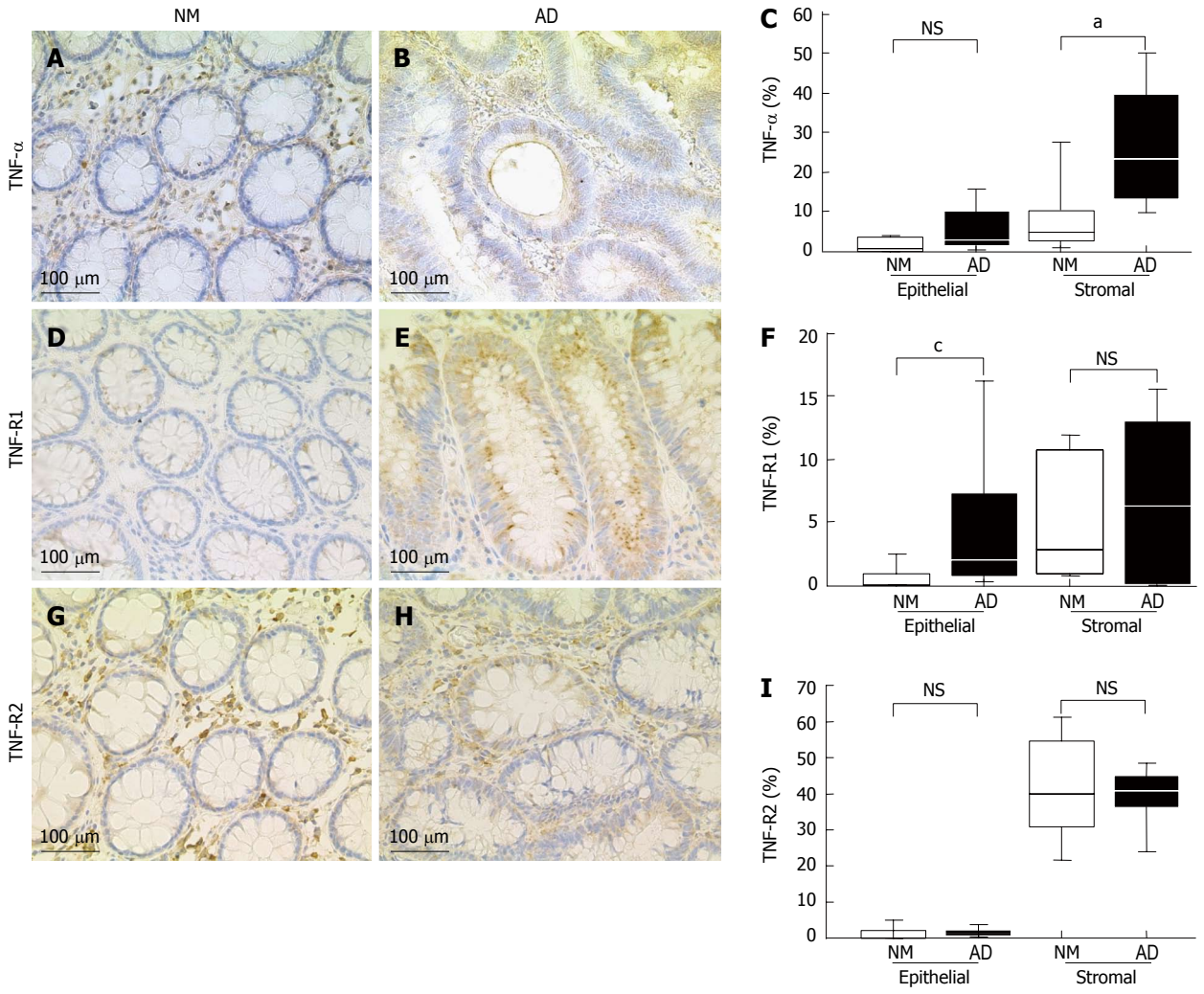


Figure 2 Immunohistochemical analyses in the normal colorectal mucosa and adenoma tissues. A: Tumor necrosis factor (TNF)-α expression in the normal colorectal mucosa; B: TNF-α expression in the adenoma tissues; C: The percentage of TNF-α-positive cells; D: Tumor necrosis factor-receptor 1 (TNF-R1) expression in the normal colorectal mucosa; E: TNF-R1 expression in the adenoma tissues; F: The percentage of TNF-R1-positive cells; G: TNF-R2 expression in the normal colorectal mucosa; H: TNF-R2 expression in the adenoma tissues; I: The percentage of TNF-R2-positive cells. Box plots display median values and interquartile ranges (C, F, I). The non-outlier range is also shown. $^{a}P < 0.05$ between NM and AD in stromal of TNF-α; $^{c}P < 0.05$ between NM and AD in epithelial of TNF-R1. NS: Non-significant; NM: Normal mucosa; AD: Adenoma.

indicator of a poor prognosis[39].

TNFR-1 is an important member of the death receptor family, which is capable of inducing apoptotic cell death[40]. In addition to its involvement in apoptotic signaling, TNF-R1 has been widely studied because it is a dual-role receptor. In addition to inducing apoptosis, it also has the ability to transduce cell survival signals. When TNF-R1 transduces cell survival signals, TNFR-associated factor 2 (TRAF-2) is recruited to the complex, which inhibits apoptosis *via* the cytoplasmic inhibitor of apoptosis protein (cIAP). The binding of TRAF-2 initiates a sequence of phosphorylation steps resulting in the activation of cFos/cJun transcription factors *via* MAPK and JNK[41]. The cFos/cJun transcription factors induce transcription of antiapoptotic, proliferative, immunomodulatory, and inflammatory genes.

TNF-α is a major mediator of cancer-related inflammation[13,42], and most of the pro-tumor actions of TNF-α appear to be mediated by TNF-R1. Mouse experiments have revealed that the development of primary cancers and metastases is attenuated in mice deficient in TNF-R1. For example, TNF-R1-/- mice are resistant to DMBA-TPA carcinogenesis, as are TNF-α-/- mice[43]. Experimental formation of lung and liver metastases was attenuated in TNF-R1-/- mice compared with that in their normal counterparts[44,45]. In wild-type mice with bone marrow cells repopulated using cells from TNF-R1-/- mice, the likelihood of colitis and colon cancer development was reduced[46] suggesting that TNF-α in the tumor microenvironment enhanced tumor development through its actions on TNF-R1-positive myeloid cells. In various pathologic states, it has been reported that the production and release of TNF-R1 may mediate host responses and determine the course and outcome of the disease by binding with TNF-α and competing with cell surface receptors.

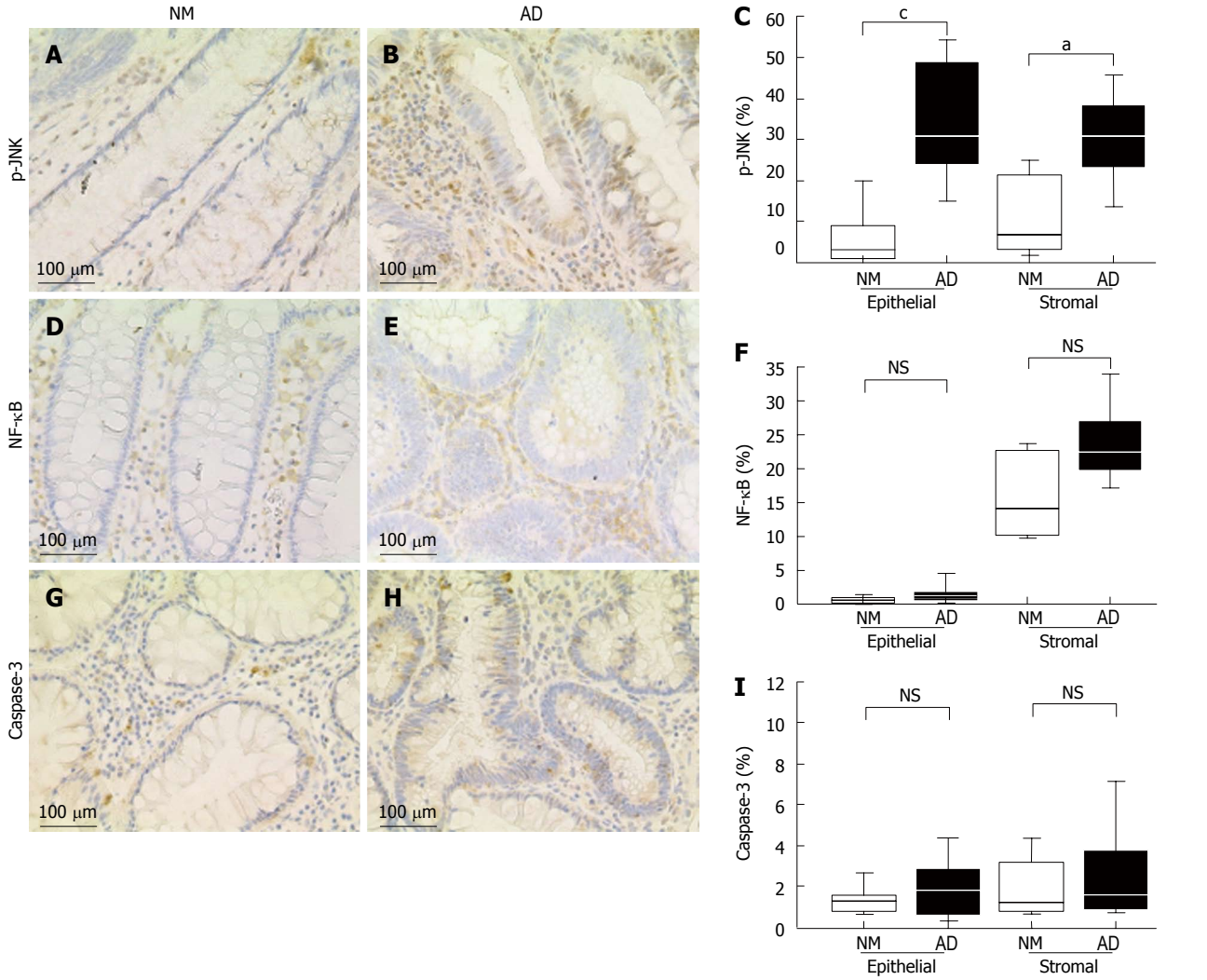


Figure 3 Immunohistochemical analyses in the normal colorectal mucosa and adenoma tissues. A: Phospho-c-Jun N-terminal kinase (p-JNK) expression in the normal colorectal mucosa; B: p-JNK expression in the adenoma tissues; C: The percentage of p-JNK positive cells; D: Nuclear factor-κ B (NF-κB) expression in the normal colorectal mucosa; E: NF-κB expression in the adenoma tissues; F: The percentage of NF-κB-positive cells; G: Caspase-3 expression in the normal colorectal mucosa; H: Caspase-3 expression in the adenoma tissues; I: The percentage of caspase-3-positive cells. Box plots display median values and interquartile ranges (C, F, I). The non-outlier range is also shown. $^{a}P < 0.05$ between NM and AD in stromal of p-JNK; $^{c}P < 0.05$ between NM and AD in epithelial of p-JNK. NS: Non-significant; NM: Normal mucosa; AD: Adenoma.

In addition to the systematic analysis of serum sTNF -R1, we also investigated, by immunohistochemical analysis, the local expressions of downstream molecules in the TNF-α signaling pathway in normal colorectal mucosa and adenoma tissues. The results revealed high levels of TNF-R1 and p-JNK expression in the epithelial cells of adenomas. Moreover, we identified co-localization of TNF-R1 and p-JNK in adenomas by immunofluorescence confocal microscopy. In the past, expression of p-JNK in adenomas has only been described by Hardwick *et al*[47]. These authors found that the expression of p-JNK was observed mainly in stromal T-lymphocytes, and epithelial cells were not stained. The discrepancy in our results was presumably due to differences in the immunohistochemical staining protocol used. The Hardwick study used phosphate- buffered saline (PBS) as the wash buffer, while we used TBS. Additionally, different antibodies were used in the two studies, which may have resulted in the absence of phospho-JNK staining in the epithelial cells of their study.

Our results suggest that the TNF-R1/p-JNK pathway is upregulated in adenomas and that this pathway may play an important role in adenoma formation, which represents the very early stages of colorectal carcinogenesis because this pathway is not upregulated in the normal colorectal mucosa. Expression of JNK in colon cancer has already received some attention[48,49]; studies have found increased activity of JNK in both rat models of colon cancer and human colorectal tumors. In addition, Zhang *et al*[50] indicated that the TNF-R1/JNK signaling cascade can functionally promote tumorigenesis of human epithelial cancers, such as squamous cell carcinomas. Moreover, a recent study conducted by our group showed that JNK/c-Jun may play an important role in promoting colorectal carcinogenesis and epithelial cell proliferation under high-fat dietary conditions[51].

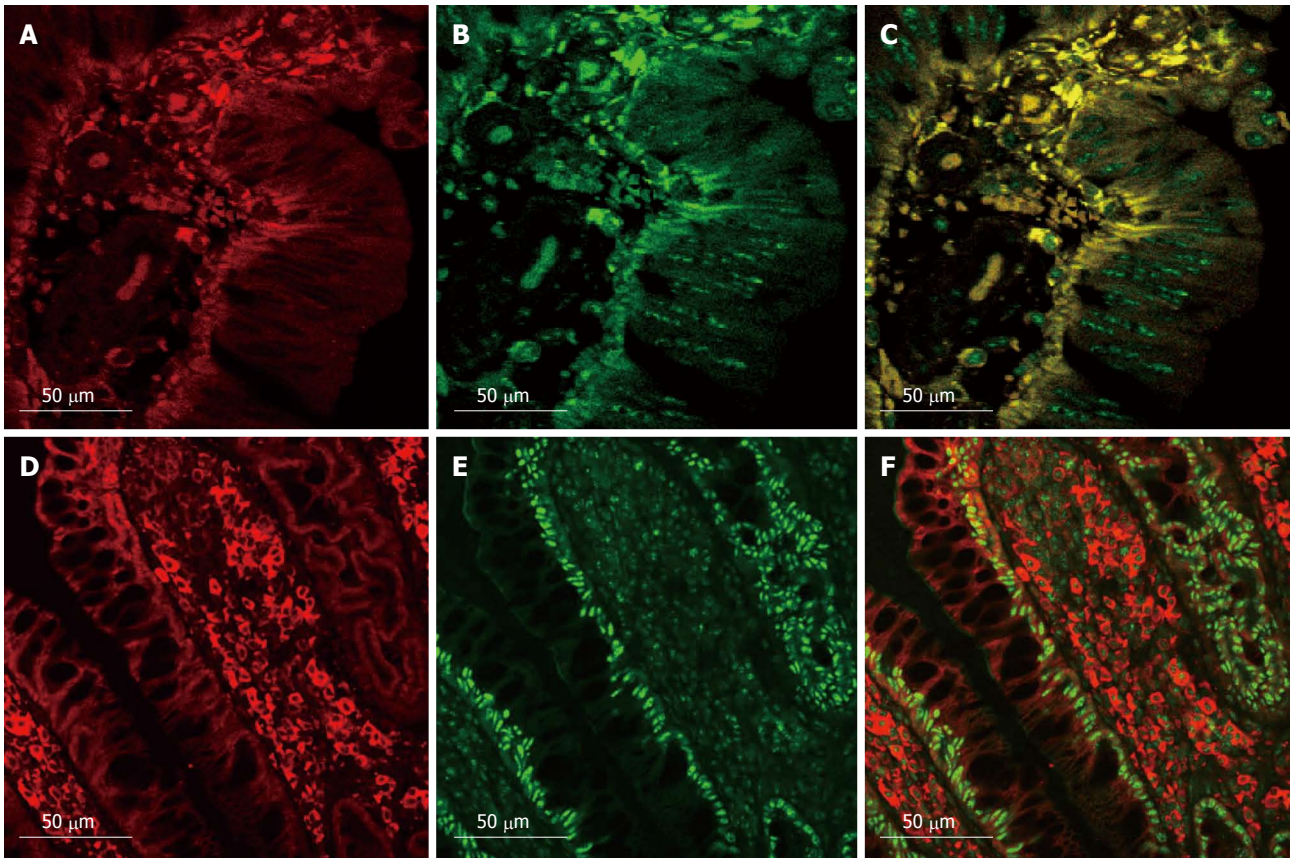


Figure 4 Confocal laser microscopic analyses of colorectal adenoma sections. A: Tumor necrosis factor receptor 1 (TNF-R1) expression; B: Phospho-c-Jun N-terminal kinase (p-JNK) expression; C: Merged image of A and B. Co-localized expression of TNF-R1 and p-JNK was noted predominantly in the epithelial cells of adenomas. Merged images showing co-localization; D: TNF-R2 expression; E: p-JNK expression; F: Merged image of D and E. Expression of TNF-R2 was localized predominantly to the stromal area of the adenoma. Merged images showed a lack of co-localization.

Our study had several important novelties. We showed for the first time that the serum levels of sTNF-R1 may have high diagnostic sensitivity and specificity as a biomarker for colorectal adenoma. Second, TNF-R1 was relatively undetectable in the normal colorectal mucosa, whereas adenomas showed high expression levels of TNF-R1. In addition, co-expression of p-JNK with TNF-R1 was observed in adenomas, although a previous study reported that the epithelial cells of adenoma showed a lack of p-JNK staining[47]. These results suggest that the TNF-R1/JNK pathway may play an important role in the development/progression of colorectal adenoma.

The present study also had some limitations. First, our research target was colorectal adenoma and not colorectal cancer. Thus, further studies targeting colorectal cancer are needed. Secondly, the control population was small. Third, different cell death receptors are able to activate JNK. Therefore, the increased p-JNK expression in adenoma tissue may be related to TNF-R1 or other effectors. The expression of cell death receptors may also be altered in inflammatory cells, which may certainly be involved in cancer progression. Fourth, it was considered that TNF-R1 may directly stimulate JNK activation, but this would also be difficult to prove.

In conclusion, this is the first study to report elevated serum levels of sTNF-R1 in patients with adenomas compared to a control group. We also found, by immunohistochemical analysis, enhanced expression of TNF-R1 in colorectal adenomas. These results suggest that TNF-R1 may not only be a promising biomarker of colorectal adenoma but also plays an important role in the very early stages of colorectal carcinogenesis. More studies are needed to elucidate the exact functions of TNF-R1 in colorectal adenomas.

ACKNOWLEDGMENTS

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COMMENTS

Background

The proinflammatory cytokine, tumor necrosis factor (TNF)-α, is a major mediator of cancer-related inflammation. However, little has been reported on the relationship between TNF-α and sporadically occurring colorectal neoplasms. Studies on human colorectal adenoma were performed to determine the expression statuses of TNF-α and its receptors.

Research frontiers

Overexpression of TNF-α and its receptors often accompanies tumor development and progression, and their important role in the pathogenesis of cancer is

now increasingly recognized.

Innovations and breakthroughs

The authors show, for the first time, that the serum levels of tumor necrosis factor-receptor 1 (TNF-R1) may have high diagnostic sensitivity and specificity as a biomarker of colorectal adenoma. TNF-R1 was hardly detected in the normal colorectal mucosa, whereas adenomas showed high expression levels of TNF-R1. In addition, co-expression of phospho-c-Jun N-terminal kinase (p-JNK) with TNF-R1 was observed in adenomas. These results suggest that the TNF-R1/JNK pathway may play an important role in the development/progression of colorectal adenoma.

Applications

TNF-R1 may be a promising biomarker of colorectal adenoma, and further studies may show that TNF-R1 expression can be used to screen for adenomas in patients as an alternative or in addition to hemoccult screening or colonoscopy screening.

Terminology

TNF-R1 belongs to the TNF receptor superfamily. In response to TNF treatment, activation of the transcription factor nuclear factor-κ B and mitogen-activated protein kinase, as well as Extracellular Signal-regulated Kinase, p38, and JNK, has been reported in most types of cells and, in some cases, apoptosis or necrosis was also induced.

Peer review

This is an interesting study investigating the importance of TNF-R1/JNK co-expression in colorectal adenoma. The major finding of the study was that serum levels of TNF-R1 were higher in patients with colorectal adenomas, while immunohistochemistry showed high expression of both TNF-R1 and p-JNK in the adenomatous tissues.

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ORIGINAL ARTICLE

Axl glycosylation mediates tumor cell proliferation, invasion and lymphatic metastasis in murine hepatocellular carcinoma

Ji Li, Li Jia, Zhen-Hai Ma, Qiu-Hong Ma, Xiao-Hong Yang, Yong-Fu Zhao

Ji Li, Zhen-Hai Ma, Xiao-Hong Yang, Yong-Fu Zhao, Department of General Surgery, the Second Affiliated Hospital of Dalian Medical University, Dalian 116027, Liaoning Province, China
Li Jia, Qiu-Hong Ma, College of Laboratory Medicine, Dalian Medical University, Dalian 116044, Liaoning Province, China
Author contributions: Li J performed the whole experiment, wrote the manuscript; Zhao YF designed the experiment, provided financial support, conducted the whole study; Jia L, Ma ZH, Ma QH and Yang XH participated in the study.
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Correspondence to: Yong-Fu Zhao, Professor, Department of General Surgery, the Second Affiliated Hospital of Dalian Medical University, 465 Zhongshan Road, Dalian 116027, Liaoning Province, China. zyf0386@sina.com
Telephone: +86-411-84671291 Fax: +86-411-84672130
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Abstract

AIM: To investigate the effects of Axl deglycosylation on tumor lymphatic metastases in mouse hepatocellular carcinoma cell lines.

METHODS: Western blotting was used to analyze the expression profile of Axl glycoprotein in mouse hepatocellular carcinoma cell line Hca-F treated with tunicamycin and PNGase F 3-(4,5)-dimethylthiazol(-zyl)-3,5-diphenyltetrazolium bromide (MTT) assay, extracellular matrix (ECM) invasion assay (*in vitro*) and tumor metastasis assay (*in vivo*) were utilized to evaluate the effect of Axl deglycosylation on the Hca-F cell proliferation, invasion and lymphatic metastasis.

RESULTS: Tunicamycin and PNGase F treatment markedly inhibited Axl glycoprotein synthesis and expression, proliferation, invasion, and lymphatic metastasis both *in vitro* and *in vivo*. In the MTT assay, proliferation was apparent in untreated Hca-F cells compared with treated Hca-F cells. In the ECM invasion assay (*in vitro*), treated cells passed through the ECMatrix gel in significantly smaller numbers than untreated cells (tunicamycin 5 μg/mL: 68 ± 8 *vs* 80 ± 9, $P = 0.0222$; 10 μg/mL: 50 ± 6 *vs* 80 ± 9, $P = 0.0003$; 20 μg/mL: 41 ± 4 *vs* 80 ± 9, $P = 0.0001$); (PNGase F 8 h: 66 ± 7 *vs* 82 ± 8, $P = 0.0098$; 16 h: 49 ± 4 *vs* 82 ± 8, $P = 0.0001$; 24 h: 34 ± 3 *vs* 82 ± 8, $P = 0.0001$). In the tumor metastasis assay (*in vivo*), average lymph node weights of the untreated Hca-F group compared with treated Hca-F groups (tunicamycin 5 μg/mL: 0.84 ± 0.21 g *vs* 0.72 ± 0.19 g, $P = 0.3237$; 10 μg/mL: 0.84 ± 0.21 g *vs* 0.54 ± 0.11 g, $P = 0.0113$; 20 μg/mL: 0.84 ± 0.21 g *vs* 0.42 ± 0.06 g, $P = 0.0008$); (PNGase F 8 h: 0.79 ± 0.15 g *vs* 0.63 ± 0.13 g, $P = 0.0766$; 16 h: 0.79 ± 0.15 g *vs* 0.49 ± 0.10 g, $P = 0.0022$; 24 h: 0.79 ± 0.15 g *vs* 0.39 ± 0.05 g, $P = 0.0001$). Also, average lymph node volumes of the untreated Hca-F group compared with treated Hca-F groups (tunicamycin 5 μg/mL: 815 ± 61 mm^3 *vs* 680 ± 59 mm^3, $P = 0.0613$; 10 μg/mL: 815 ± 61 mm^3 *vs* 580 ± 29 mm^3, $P = 0.0001$; 20 μg/mL: 815 ± 61 mm^3 *vs* 395 ± 12 mm^3, $P = 0.0001$); (PNGase F 8 h: 670 ± 56 mm^3 *vs* 581 ± 48 mm^3, $P = 0.0532$; 16 h: 670 ± 56 mm^3 *vs* 412 ± 22 mm^3, $P = 0.0001$; 24 h: 670 ± 56 mm^3 *vs* 323 ± 11 mm^3, $P = 0.0001$).

CONCLUSION: Alteration of Axl glycosylation can attenuate neoplastic lymphatic metastasis. Axl N-glycans may be a universal target for chemotherapy.

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Key words: Axl; Glycosylation; Hepatocellular carcinoma; Lymphatic metastasis

Peer reviewer: Francesco Feo, Professor, Department of Biomedical Sciences, Section of Experimental Pathology and Oncol-

ogy, University of Sassari, Via P, Manzella 4, 07100 Sassari, Italy

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INTRODUCTION

The receptor tyrosine kinases (RTKs) constitute a large family of transmembrane proteins that relay signals from extracellular growth factors into the cell[1,2]. The Tyro-Axl-Mer (TAM) subfamily shares the vitamin K-dependent ligand Gas6 (growth arrest specific 6). TAM receptors contain a combination of two immunoglobin-like domains and dual fibronectin type Ⅲ repeats in the extracellular region, and a cytoplasmic kinase domain[3,4]. The TAM receptors regulate a diverse range of cellular responses including cell survival, proliferation, autophagy, migration, angiogenesis, platelet aggregation, and natural killer cell differentiation[4].

The Axl receptor (also called UFO, Tyro7, and Ark) is a RTK originally identified as a transforming gene in chronic myeloid leukemia[5,6]. Axl is expressed in various organs, including the brain, suggesting its involvement in mesenchymal and neural development[7,8]. Axl has been shown to have transforming potential when overexpressed during development. Axl overexpression is clearly associated with invasiveness and metastasis in several cancer cell types, including myeloid leukemia[6,9], esophageal[10], metastatic lung[11], metastatic colon[12], renal cell[13], prostate[14], breast[15], gastric[16], and thyroid[17] cancers. Axl also affects multiple pathways in angiogenesis[11]. Thus, Axl may play an important role in tumor progression, although its mechanism remains unknown.

Protein glycosylation is one of the major types of posttranslational modifications that has profound biological implications[18,19]. Specific changes in the glycosylation pattern of cell surface glycoproteins have been shown to correlate with metastatic efficiency in tumor cells[20]. In particular, protein N-glycosylation is one of the most prominent biochemical alterations in tumorigenesis and metastatic spread[21,22]. A cell surface transmembrane glycoprotein, little is known about the mechanism of Axl deglycosylation.

The mouse hepatocellular carcinoma cell line Hca-F is highly aggressive, with a metastasis rate over 80%. Hca-P, on the other hand, has a lymphatic metastasis rate of less than 30%. Both cell lines are derived from 615-mice ascites-type hepatocellular carcinoma cells. Hca-F and Hca-P cells metastasize only to lymph nodes, and not extrahepatic organs. However, the relationship between Axl glycosylation and lymphatic metastasis of mouse hepatocellular carcinoma cells remains unclear.

Our aim was to investigate whether Axl glycosylation regulates lymphatic metastasis. We demonstrated a possible correlation, based upon regulation of Axl glycosylation in mouse hepatocellular carcinoma cells.

MATERIALS AND METHODS

Cell culture and animals

Mouse hepatocellular carcinoma cell lines Hca-F and Hca-P, grown and stored in our institution (Department of Pathology, Dalian Medical University) were cultured in 90% Roswell Park Memorial Institute (RPMI)-1640 (Gibco) and supplemented with antibiotics (1 × penicillin/streptomycin 100 U/mL, Gibco) and 10% fetal bovine serum (FBS) (Gibco). Cells were incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2. 615-mice (8 wk old males) were obtained from the Experimental Animal Center of Dalian Medical University.

Whole protein extract

10^7 cells were centrifuged at room temperature at 1000 × *g* for 10 min. Cells were rinsed twice with phosphate buffered saline (PBS) at 1000 × *g* for 5 min, and lysed with a protease inhibitor cocktail (whole protein extraction kit KGP2100, KeyGEN). Cells were suspended on a swing bed at 4 ℃ for 15 min, and centrifuged at 4 ℃ at 14 000 × *g* for 15 min. Protein concentration of the whole cells was measured with a bicinchoninic acid protein assay kit (KGPBCA, KeyGEN).

Western blotting analysis

Western blotting analysis was performed to evaluate Axl (with or without tunicamycin or PNGase F treatment) protein levels. Extracted proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes (Pall Corporation). After blocking for 2 h with 5% skimmed milk in PBS containing 0.1% Tween 20 (PBST), membranes were incubated with rabbit anti-mouse Axl polyclonal antibody (Santa Cruz Biotech Inc., 1/200 diluted) overnight in 5% powdered skim milk buffer, washed thrice with PBS with 0.1% Tween 20, and then incubated with secondary antibody anti-rabbit-HRP (Santa Cruz Biotech Inc., 1/3000 diluted). Glyceraldehyde-3-phosphate dehydrogenase antibody (Santa Cruz Biotech Inc., 1/200 diluted) was used as controls. All blot analysis was performed with a ECL Western blotting kit (Amersham Biosciences, United Kingdom).

Tunicamycin treatment

To inhibit N-linked glycosylation of newly synthesized proteins, Hca-F cells were washed once with PBS and cultivated for 12 h in fresh culture media (90% RPMI 1640 supplemented with antibiotics) with or without tunicamycin (Sigma Aldrich, St. Louis, MO) in a dose-dependent manner (0 μg/mL, 5 μg/mL, 10 μg/mL, or 20 μg/mL). Cells were washed with PBS and subjected to Western blotting analysis, 3-(4,5)-dimethylthiazol(-zyl)-3,5-diphenyltetrazolium bromide (MTT), migration *in*

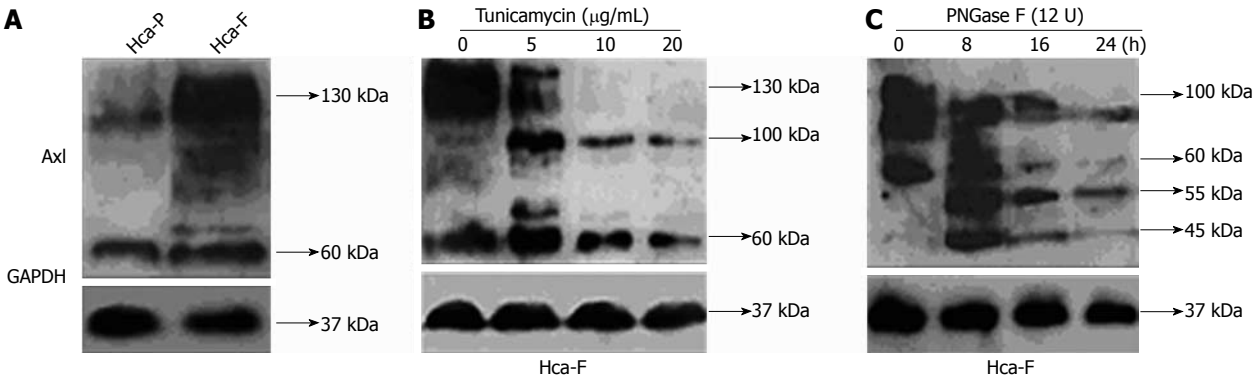


Figure 1 Expression profile of Axl glycoprotein in mouse hepatocellular carcinoma cell lines. A: Axl glycoprotein levels by Western blotting analysis in Hca-P and Hca-F cell lines. Relative signal intensities of Axl protein were compared with GAPDH by LabWorks (TM ver4.6, UVP; BioImaging Systems), ($^{a}P < 0.05$ *vs* untreated Hca-F cells); B: Hca-F cells were treated with 0 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL tunicamycin for 12 h. Total protein extracts were loaded for each sample; C: Hca-F cell protein was deglycosylated with 12 units of PNGase F in lysis buffer. Probes were incubated at 37 ℃ in a time-dependent manner (0 h, 8 h, 16 h, 24 h). Protein was separated on a gel for Western blotting Analysis. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane, and were detected by rabbit anti-mouse Axl polyclonal antibody. GAPDH blotting was used as the control. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

vitro, and tumor metastasis *in vivo* assays.

PNGase F treatment

To remove N-glycans, protein fractions (100 μg) from Hca-F cells were deglycosylated with 25 units of PNGase F (Elizabethkingia meningoseptica; Sigma Aldrich, St. Louis, MO) in lysis buffer. Probes were incubated for 8 h, 16 h and 24 h at 37 ℃. The reaction was terminated with Laemmlie's sample buffer and proteins were separated on a gel as described earlier.

For deglycosylation of membrane proteins, intact Hca-F cells were incubated with 25 units of PNGase F for 24 h, washed, and treated as described for the MTT, migration *in vitro*, and tumor metastasis *in vivo* assays.

3-(4,5)-dimethylthiazol(-zyl)-3,5-diphenyltetrazolium bromide assay

10^6 cells in 200 μL RPMI 1640 were seeded in duplicate into 96-well culture plates, and 100 μL MTT (5 mg/mL, Sigma) was added at 24 h, 48 h, 72 h, 96 h, and 120 h, respectively. After 4 h incubation at 37 ℃ in 5% CO_2, 100 μL/well DMSO (final concentration 25%, Gibco) was pipetted to solubilize the formazan product for 30 min at room temperature. Absorbency (490 angstroms) was measured using a microplate reader (Bio-Rad).

In vitro extracellular matrix invasion assays

Cell invasion *in vitro* was demonstrated using 24-well transwell units (Corning, NY, United States) with a 8 μm pore size polycarbonate filter coated with ECMatrix gel (Chemicon) to form a continuous thin layer[23]. Cells (3×10^5) were harvested in serum-free medium containing 0.1% BSA and added to the upper chamber. The lower chamber contained 500 μL RPMI 1640. Cells were incubated for 24 h at 37 ℃, 5% CO_2 incubator. At the end of incubation, cells on the upper surface of the filter were completely removed with a cotton swab. The filters were fixed in methanol and stained with Wright-Giemsa. Cells invading the matrigel that reached the lower surface of the filter were counted with light microscopy at a magnification of 400 ×. Samples were acquired in triplicate and data expressed as the average cell number in 5 fields.

In vivo tumor metastasis assay

Forty eight 615-mice were provided with sterilized food and water and equally divided into eight groups. 10^7 Hca-F cells (with or without tunicamycin or PNGase F treatment) were subcutaneously inoculated into the footpads. After 3 wk, mice were sacrificed and their axillary lymph nodes were isolated, weighed, and photographed.

Statistical analysis

Each assay was performed at least three times. Data were presented as the mean ± SD. Statistical differences between test groups was assessed by one-way analysis of variance and Scheffe's test for post hoc analysis. A *P*-value of less than 0.05 was considered statistically significant. SPSS version 13.0 software was used for statistical analysis.

RESULTS

Expression profile of Axl glycoprotein in mouse hepatocellular carcinoma cell lines

Axl glycoprotein relative expression was determined by Western blotting analysis using whole-cell extracts (Figure 1A). Axl expression varied among cell lines, with higher and lower levels in Hca-F and Hca-P cells, respectively (Figure 1A, $P < 0.05$).

Tunicamycin, an inhibitor of endogenous N-linked glycosylation of newly synthesized proteins, was used to inhibit Axl glycosylation of Hca-F cells. Treatment in a dose dependent manner (0 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL) for 12 h showed N-linked glycosylation to be highly sensitive to tunicamycin inhibition (Figure 1B).

Axl appears as broad bands, with molecular weights ranging from 60 kDa to 140 kDa. With tunicamycin treatment, 130 kDa Axl band density decreased, 60 kDa

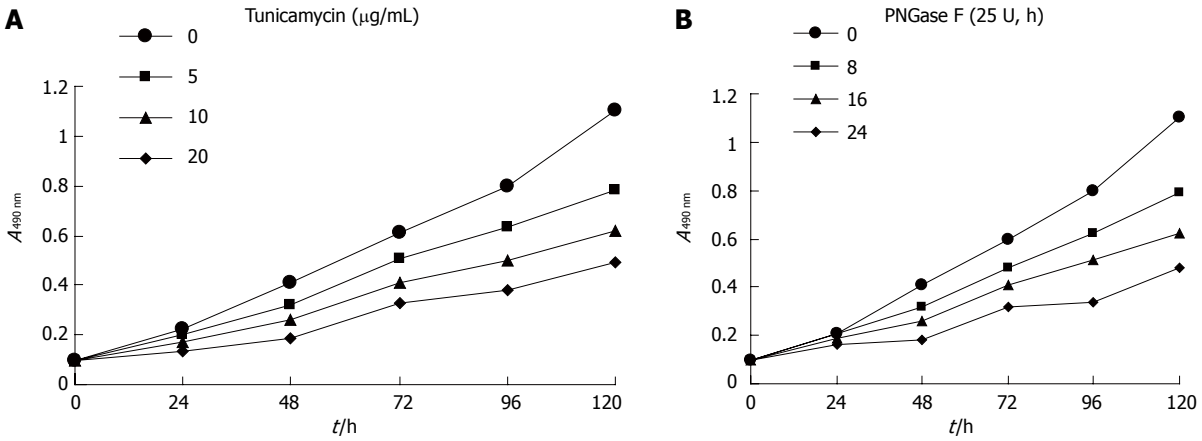


Figure 2 Axl deglycosylation effects on cell proliferation *in vitro*. Hca-F cells were exposed to tunicamycin or PNGase F and harvested at 24 h, 48 h, 72 h, 96 h, and 120 h. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Decreased proliferative ability was detected in cells treated with tunicamycin (A) or PNGase F (B), compared with untreated Hca-F cells. Data was obtained in triplicate.

band density increased, and a 100 kDa band appeared. As the dose of tunicamycin increased, the 130 kDa Axl band completely disappeared.

Whole protein aliquots extracted from Hca-F cells were exposed to exogenous PNGase F for deglycosylation (Figure 1C). 55 kDa and 45 kDa Axl bands appeared with PNGase F treatment. However, 60-140 kDa Axl band density significantly decreased. These results suggest that the N-glycosylation process for Hca-F cells responded to tunicamycin and PNGase F treatment.

Axl deglycosylation reduces cell proliferation in vitro

Hca-F cells treated with tunicamycin or PNGase F were measured for proliferative activity. Proliferation was apparent in untreated Hca-F cells compared with treated Hca-F cells (Figure 2A, B). Thus, Axl deglycosylation inhibited Hca-F cell proliferation *in vitro*.

Axl deglycosylation alters invasiveness of Hca-F cells in vitro

To examine whether Axl deglycosylation affects invasiveness of Hca-F cells, we performed *in vitro* ECMatrix gel analysis. We found that untreated and tunicamycin (Figure 3A) or PNGase F (Figure 3B) treated Hca-F cells or PNGase F had differing abilities to pass through an ECMatrix coated filter; therefore, the numbers of invading cells were unequal. Treated cells with tunicamycin passed through the ECMatrix gel in significantly smaller numbers than untreated cells (5 μg/mL: 68 ± 8 *vs* 80 ± 9, $P = 0.0222$; 10 μg/mL: 50 ± 6 *vs* 80 ± 9, $P = 0.0003$; 20 μg/mL: 41 ± 4 *vs* 80 ± 9, $P = 0.0001$). Similar results were shown with PNGase F treated Hca-F cells at 8 h, 16 h, and 24 h compared with untreated cells (66 ± 7 *vs* 82 ± 8, $P = 0.0098$; 49 ± 4 *vs* 82 ± 8, $P = 0.0001$; 34 ± 3 *vs* 82 ± 8, $P = 0.0001$). These results indicated that Axl deglycosylation reduced the invasiveness of Hca-F cells *in vitro*.

Axl deglycosylation inhibits the metastatic ability of Hca-F cells to peripheral lymph nodes in vivo

To further evaluate whether Axl deglycosylation was essential for tumor lymphatic metastasis *in vivo*, we tested the effect of Axl deglycosylation on the metastatic ability of Hca-F cells in mice peripheral lymph nodes. Treated and untreated Hca-F cells were injected in the footpads of 615-mice. After 3 wk' inoculation, a significant reduction in positive lymph nodes in the deglycosylation groups was observed, compared with untreated controls (Figure 4). Average lymph node weights of the untreated Hca-F group compared with dose-adjusted tunicamycin treated Hca-F groups (5 μg/mL: 0.84 ± 0.21 g *vs* 0.72 ± 0.19 g, $P = 0.3237$; 10 μg/mL: 0.84 ± 0.21 g *vs* 0.54 ± 0.11 g, $P = 0.0113$; 20 μg/mL: 0.84 ± 0.21 g *vs* 0.42 ± 0.06 g, $P = 0.0008$) (Figure 4A left). The average lymph node volumes of these groups were 815 ± 61 mm^3 *vs* 680 ± 59 mm^3, $P = 0.0613$; 815 ± 61 mm^3 *vs* 580 ± 29 mm^3, $P = 0.0001$; 815 ± 61 mm^3 *vs* 395 ± 12 mm^3, $P = 0.0001$ (Figure 4A right).

The average lymph node weights in the untreated Hca-F compared with PNGase F treated groups were: 8 h: 0.79 ± 0.15 g *vs* 0.63 ± 0.13 g, $P = 0.0766$; 16 h: 0.79 ± 0.15 g *vs* 0.49 ± 0.10 g, $P = 0.0022$; 24 h: 0.79 ± 0.15 g *vs* 0.39 ± 0.05 g, $P = 0.0001$ (Figure 4B left). The average lymph node volumes of these groups were: 670 ± 56 mm^3 *vs* 581 ± 48 mm^3, $P = 0.0532$; 670 ± 56 mm^3 *vs* 412 ± 22 mm^3, $P = 0.0001$; 670 ± 56 mm^3 *vs* 323 ± 11 mm^3, $P = 0.0001$ (Figure 4B right). These results demonstrate Axl deglycosylation may reduce Hca-F cells to peripheral lymph nodes *in vivo*.

DISCUSSION

Axl has garnered attention because of its high expression in many tumor cells, and its key role in neoplastic invasion and metastasis. In this study, we demonstrated Axl protein expression varied based on antineoplastic treatment of mouse hepatocellular carcinoma cell lines Hca-F and Hca-P. We found Axl protein expression to be higher in Hca-F cells, which have high lymphatic metastasis potential compared with Hca-P cells, which have low lymphatic metastasis potential. This confirms previ-

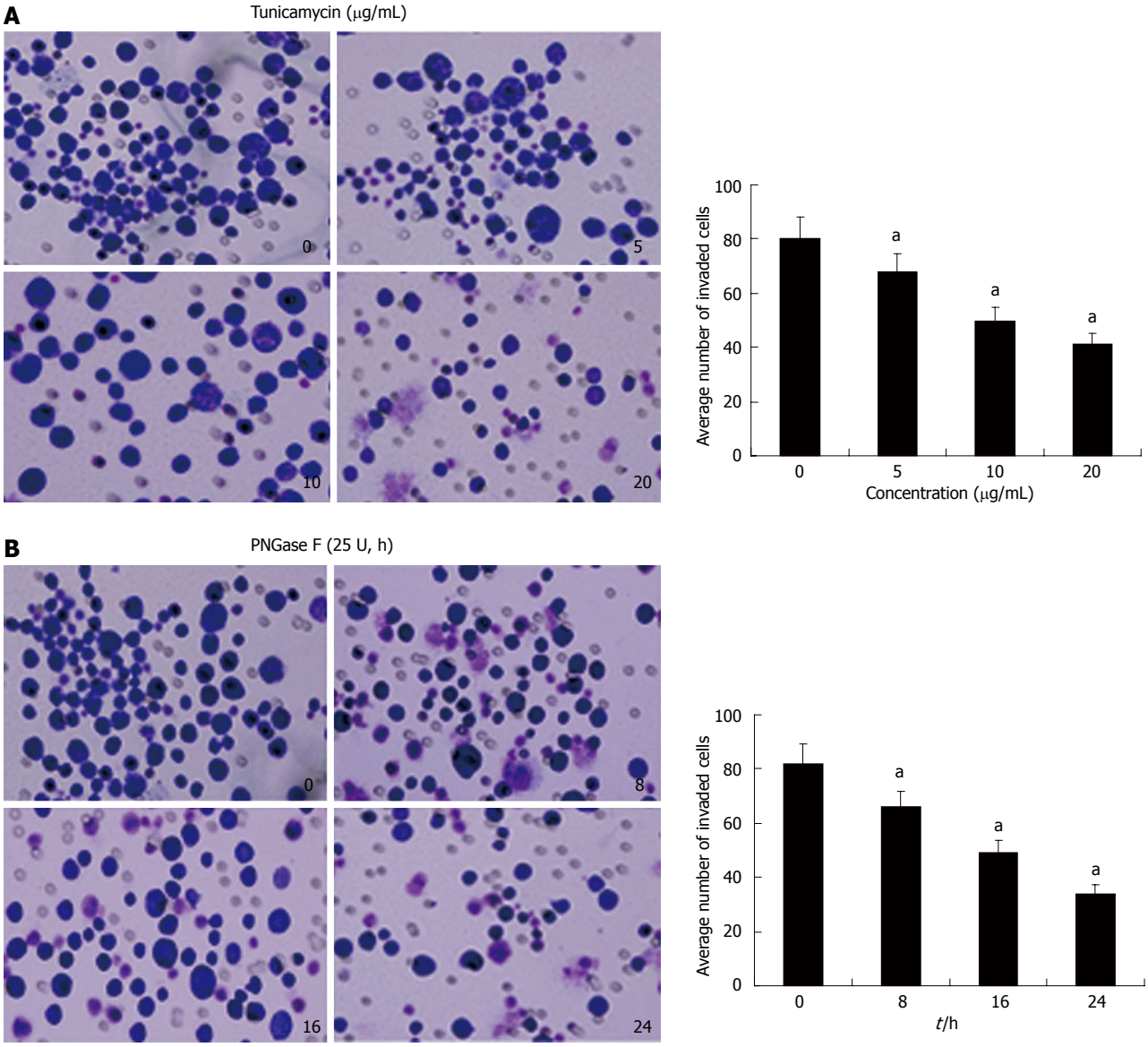


Figure 3 Axl deglycosylation alters the invasive ability of Hca-F cells *in vitro*. *In vitro* ECMatrix gel analysis. Wright-Giemsa staining results of the lower surface filter showed that the cells passed through the filter and attached to the lower side (400 ×). The average number of cells invading the filter was counted. Cells treated with tunicamycin (A) or PNGase F (B) were significantly less invasive ($^aP < 0.05$ *vs* untreated Hca-F cells) than untreated Hca-F cells. Data was obtained in triplicate.

ously reported findings of Axl overexpression in highly invasive lung adenocarcinoma cell lines, compared with their less invasive counterparts[11]. This suggests that high Axl expression may be associated with tumor lymphatic metastasis, and that Axl may be associated with tumor metastatic potential.

In our study, we achieved Hca-F deglycosylation with two methods. First, we inhibited N-glycan biosynthesis with tunicamycin; secondly, we extracted protein in the presence of PNGase F enzyme, which digests N-glycans. Both treatments resulted in significant effects on cell surface N-glycans by Western blotting assays.

Among post-translational modification reactions involving proteins, glycosylation is the most common; nearly 50% of all proteins are glycosylated[24]. Alterations of glycan structures are frequently observed in various cancer cells[25]; and this appears to be one association in cancer invasion and metastasis. We found Axl deglycosylation to be a possible factor in tumor progression, including cell proliferation, invasion, and lymphatic metastasis. In this study, we detected a significant inhibition of proliferation and invasion in Axl deglycosylated Hca-F cells *in vitro*, by both MTT and extracellular matrix assays. These results confirmed prior reports that cell proliferation requires growth factors signalling through cell surface glycoprotein receptors, which may be inactive when underglycosylated[26]. Although our findings support the role of Axl deglycosylation in reducing cell proliferation and invasion *in vitro*, its mechanism had not been elucidated. Further experiments showed that Axl deglycosylation led to a significant reduction in metastatic lymph node burden *in vivo*. These results were consistent previous reports of changes in N-linked oligosaccharide branching associated with malignancy and metastasis[27].

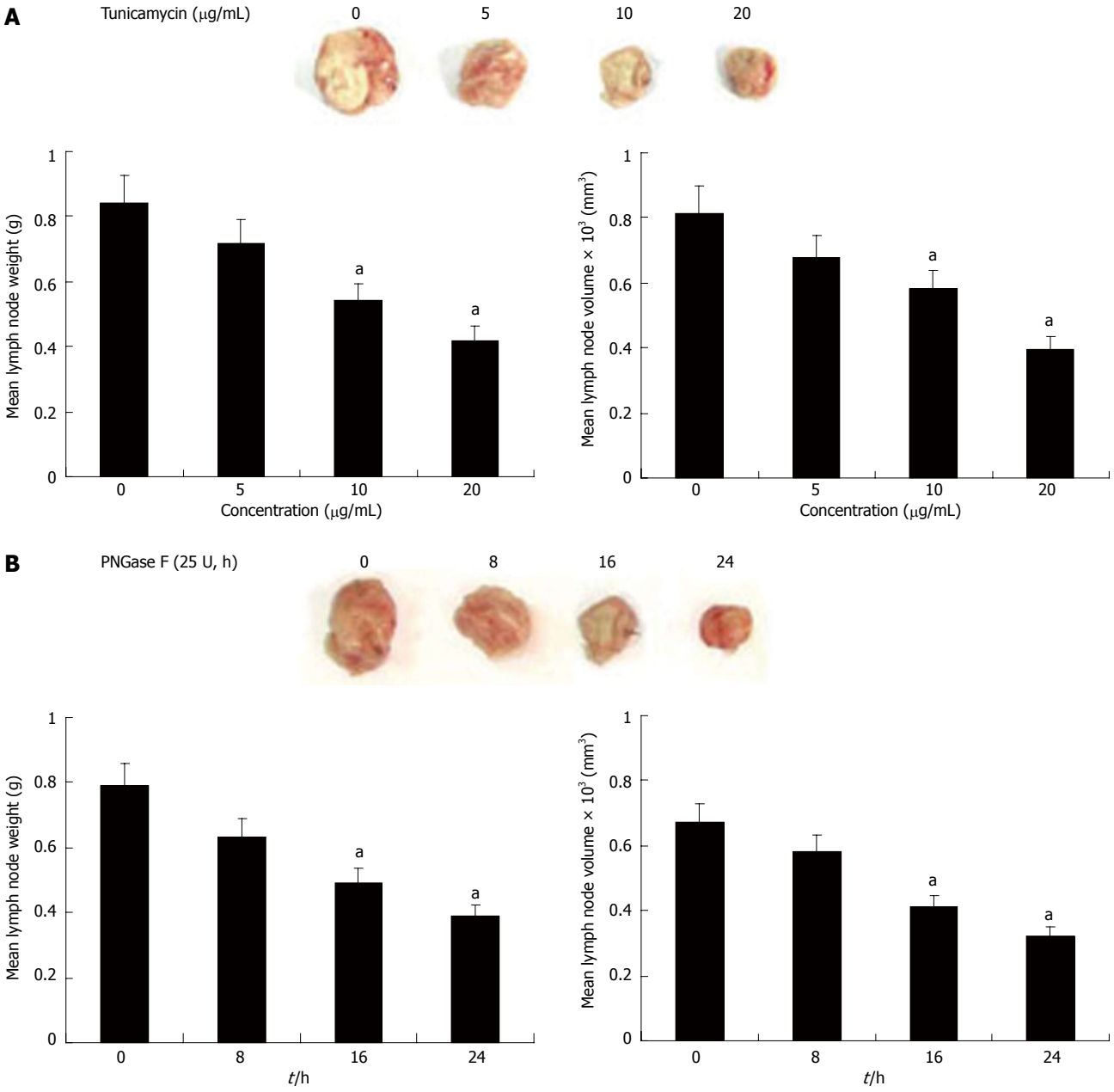


Figure 4 Axl deglycosylation inhibits the ability of Hca-F cells to metastasize to peripheral lymph nodes *in vivo*. Untreated and treated Hca-F cells were injected into the footpads of 615-mice. After 3 wk' inoculation, the mice were sacrificed and axillary lymph nodes isolated, weighed, measured, and photographed. A significant reduction in mean tumor weight ($n = 6$) of Axl tumor deglycosylation was observed, as compared with untreated Hca-F cells ($^aP < 0.05$ *vs* untreated Hca-F cells).

Some authors reported that the addition of exogenous Gas6 mediated the migration and invasion of Hca-F cells both *in vitro* and *in vivo* through the Axl pathway[28]. RNAi-mediated knockdown of Axl expression decreased the ability of YAP-expressing MIHA cells and of the primary HCC cell line to proliferate and invade[29]. In our study, we were unable to elucidate the mechanism by which Axl deglycosylation inhibits lymphatic metastasis in murine Hca-F cells. However, in many glycoproteins, N-linked oligosaccharides contribute to the folding, stability, and biological function of adhesion molecules and growth factor receptors on cell surfaces[30-32]. An increasing body of evidence indicates that glycoprotein glycans are involved in the regulation of cellular functions, including cell-cell communication and signal transduction[33,34]. The products of N-acetylglucosaminyltransferase (GnT)-IV, GnT-V and 1,6-fucosyltransferase (1,6-FucT) are all increased in hepatocellular carcinoma[35]. The presence of 1,6-GlcNAc structures in N-glycans and the expression of GnT-V, which catalyzes the addition of the 1,6-branching, were shown to promote metastasis[36-39]. At the very least, these reports demonstrate the relationship between metastasis and N-glycans to be extremely complicated. This area requires additional research.

In conclusion, we have found a role of Axl glycosylation in mediating tumor cell proliferation and invasion, and have provided the first evidence that Axl deglycosylation is required for lymphatic metastasis in murine

hepatocellular carcinoma cell lines. These results may at least partially explain the role of Axl glycosylation in the promotion of lymphatic metastasis. This study may provide new insights into regulatory mechanisms of mouse hepatocellular carcinoma with lymphatic metastasis.

COMMENTS

Background

Axl has been shown to have transforming capability when overexpressed. Prior studies have revealed Axl overexpression to be clearly associated with cancer invasiveness and metastasis. Axl also has multiple effects in angiogenesis. While Axl may play an important role in tumor progression, its mechanisms of action have not been understood.

Research frontiers

The authors investigated the potential effect of Axl deglycosylation the regulation of tumor lymphatic metastasis in mouse hepatocellular carcinoma cell lines. The authors evaluated the expression profile of Axl glycoprotein in the mouse hepatocellular carcinoma cell line Hca-F, which was treated with tunicamycin and PNGase F. Furthermore, the authors analyzed the effect of Axl glycosylation by tunicamycin and PNGase F treatment in Hca-F cells with regards to proliferation, invasion, and lymphatic metastasis both *in vitro* and *in vivo*.

Innovations and breakthroughs

Protein N-glycosylation is increasingly being recognized as one of the most prominent biochemical alterations in tumorigenesis and metastatic spread. However, as a cell surface transmembrane glycoprotein, little is known about Axl deglycosylation and its mechanism of action. Axl glycosylation was attenuated by tunicamycin and PNGase F to determine the effect on Hca-F cell proliferation, invasion, and lymphatic metastasis.

Applications

The authors have found the role of Axl glycosylation in mediating tumor cells proliferation, invasion and provided the first evidence that deglycosylation of Axl is required for metastasis of hepatocellular carcinoma cells to lymph nodes. This study may provide new insights into regulatory mechanisms of mouse hepatocellular carcinoma with lymphatic metastasis.

Terminology

The Axl receptor (also named UFO, Tyro7, and Ark) is a receptor tyrosine kinase (RTK) originally identified as a transforming gene in chronic myeloid leukemia. The RTKs constitute a large family of transmembrane proteins that relay signals from extracellular growth factors into the cell.

Peer review

In this study, the effect of Axl deglycosylation on lymphatic metastasis was investigated in mouse hepatocellular carcinoma cell lines. Differing Axl expression levels were found in mouse Hca-F and Hca-P cell lines, which are characterized by high and low metastatic potential, respectively. A decrease in Axl glycosylation by tunicamycin or PNGase F treatment resulted in a reduced proliferation, invasion, and lymphatic metastasis, both *in vitro* and *in vivo*. This work is potentially relevant in understanding hepatocellular carcinoma.

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ORIGINAL ARTICLE

Protection of ghrelin postconditioning on hypoxia/reoxygenation in gastric epithelial cells

Zhang-Bo Liu, Su-Juan Fei, Sheng-Ping Zhu, Jin-Zhou Zhu, Hong-Xia Han, Qiu-Ju Dong, Jian-Fu Zhang

Zhang-Bo Liu, Su-Juan Fei, Sheng-Ping Zhu, Jin-Zhou Zhu, Hong-Xia Han, Qiu-Ju Dong, Department of Gastroenterology, The Affiliated Hospital of Xuzhou Medical College, 99 West Huaihai Road, Xuzhou 221002, Jiangsu Province, China
Jian-Fu Zhang, Department of Physiology, Xuzhou Medical College, Xuzhou 221002, Jiangsu Province, China
Author contributions: Fei SJ and Zhang JF designed the research; Liu ZB, Han HX and Dong QJ performed the research; Zhu SP contributed to the analysis of new reagents; Zhu JZ drafted the paper.
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Correspondence to: Jian-Fu Zhang, Professor, Department of Physiology, Xuzhou Medical College, 84 West Huaihai Road, Xuzhou 221002, Jiangsu Province, China. jfzhang@xzmc.edu.cn
Telephone: +86-516-83262105 Fax: +86-516-85582071
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Abstract

AIM: To investigate the protective effect and mechanisms of ghrelin postconditioning against hypoxia/reoxygenation (H/R)-induced injury in human gastric epithelial cells.

METHODS: The model of H/R injury was established in gastric epithelial cell line (GES-1) human gastric epithelial cells. Cells were divided into seven groups: normal control group (N); H/R postconditioning group; DMSO postconditioning group (DM); ghrelin postconditioning group (GH); D-Lys3-GHRP-6 + ghrelin postconditioning group (D + GH); capsazepine + ghrelin postconditioning group (C + GH); and LY294002 + ghrelin postconditioning group (L + GH). 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to detect GES-1 cell viability. Hoechst 33258 fluorochrome staining and flow cytometry were conducted to determine apoptosis of GES-1 cells. Spectrophotometry was performed to determine release of lactate dehydrogenate (LDH). Protein expression of Bcl-2, Bax, Akt, and glycogen synthase kinase (GSK)-3β was determined by western blotting. Expression of vanilloid receptor subtype 1 (VR1), Akt and GSK-3β was observed by immunocytochemistry.

RESULTS: Compared with the H/R group, cell viability of the GH group was significantly increased in a dose-dependent manner (55.9% ± 10.0% *vs* 69.6% ± 9.6%, 71.9% ± 17.4%, and 76.3% ± 13.3%). Compared with the H/R group, the percentage of apoptotic cells in the GH group significantly decreased (12.38% ± 1.51% *vs* 6.88% ± 0.87%). Compared with the GH group, the percentage of apoptotic cells in the D + GH group, C + GH group and L + GH groups significantly increased (11.70% ± 0.88%, 11.93% ± 0.96%, 10.20% ± 1.05% *vs* 6.88% ± 0.87%). There were no significant differences in the percentage of apoptotic cells between the H/R and DM groups (12.38% ± 1.51% *vs*13.00% ± 1.13%). There was a significant decrease in LDH release following ghrelin postconditioning compared with the H/R group (561.58 ± 64.01 U/L *vs* 1062.45 ± 105.29 U/L). There was a significant increase in LDH release in the D + GH, C + GH and L + GH groups compared with the GH group (816.89 ± 94.87 U/L, 870.95 ± 64.06 U/L, 838.62 ± 118.45 U/L *vs* 561.58 ± 64.01 U/L). There were no significant differences in LDH release between the H/R and DM groups (1062.45 ± 105.29 U/L *vs* 1017.65 ± 68.90 U/L). Compared with the H/R group, expression of Bcl-2 and Akt increased in the GH group, whereas expression of Bax and GSK-3β decreased. Compared with the GH group, expression of Bcl-2 decreased and Bax increased in the D + GH, C + GH and L + GH groups, and Akt decreased and GSK-3β increased in the L + GH group. The H/R group also upregulated expression of VR1 and GSK-3β and downregulated Akt. The number of VR1-positive and Akt-positive cells in the GH group significantly increased, whereas the number of GSK-3β-positive cells significantly decreased. These effects of ghrelin were

reversed by capsazepine and LY294002.

CONCLUSION: Ghrelin postconditioning protected against H/R-induced injury in human gastric epithelial cells, which indicated that this protection might be associated with GHS-R, VR1 and the PI3K/Akt signaling pathway.

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Key words: Human gastric epithelial cells; Ghrelin; Pharmacological postconditioning; Hypoxia/reoxygenation; Apoptosis

Peer reviewer: Yuji Naito, Professor, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

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INTRODUCTION

Gastric ischemia-reperfusion (GI/R) injury is a common clinical pathophysiological process. In case of clinical multiple organ dysfunction syndrome, gastrointestinal mucosal ischemia due to the redistribution of blood is the earliest to occur, and is more obvious than other organ ischemia-reperfusion (I/R) injury. Thus, the stomach is considered the earliest organ to be involved[1,2]. In recent years, ischemic postconditioning (Ipost) has been discovered to be an important endogenous protective mechanism and hypoxia postconditioning (HPost) can be elicited after hypoxia and reoxygenation in cell culture[3-5]. It has been demonstrated that IPost can effectively reduce myocardial injury[6]. Pharmacological postconditioning is the extension of Ipost, in which a drug is applied to the ischemic myocardium or hypoxic cardiomyocytes during the first few minutes of reperfusion or reoxygenation, significantly reducing organ reperfusion injury[7].

Ghrelin is a 28-amino-acid peptide, which was initially identified from rat stomach[8]. In humans, it acts as the endogenous ligand for the growth hormone secretagogue receptor (GHSR)[9]. GHSR, a seven transmembrane-domain G-protein coupled receptor was cloned and identified as the receptor for a family of synthetic ligands. GHSR has been observed in the human gastrointestinal tract[10]. Ghrelin is predominantly produced in the stomach by endocrine cells of the fundic mucosa, formerly known as X/A-like cells[9,11,12]. Several studies have suggested that the many physiological actions are associated with ghrelin, including neuroendocrine, cardiovascular and gastrointestinal functions[13,14]. Ghrelin plays an important role in mucosal defense, such as resistance against a variety of ulcerogenic stimuli, including ethanol, stress and I/R[15-17].

Vanilloid receptor subtype 1 (VR1) is a nonselective cation channel, primarily expressed in central and peripheral terminals of nonmyelinated primary afferent neurons[18]. VR1 may be activated by physical and chemical mediators that contain noxious thermal stimulation, noxious protons, and vanilloid compounds such as capsaicin[19,20]. Our study aimed to establish whether the effect of ghrelin was mediated by VR1, and whether ghrelin served as a new activator of VR1.

The phosphoinositide 3-kinase (PI3K/Akt) signaling pathway has been implicated in the control of major cellular responses including cell proliferation, survival, development, differentiation, cell cycle, and apoptosis[21,22]. The PI3K/Akt signaling pathway is a type of significant antiapoptotic factor that plays an important protective role by reducing apoptosis-associated protein kinase caspase family, Bcl-2 and other apoptotic factors[23].

The primary aim of this study was to investigate whether ghrelin postconditioning had a protective effect on hypoxia/reoxygenation (H/R) injury in human gastric epithelial cells, and whether GHSR, VR1 and the PI3K/Akt signaling pathway are involved in this protection.

MATERIALS AND METHODS

Materials

The materials and reagent were as follows: human gastric epithelial cell line (GES-1) from Beijing Cancer Hospital; ghrelin, D-Lys3-GHRP-6, capsazepine and LY294002 (Sigma-Aldrich, United States) were dissolved in 100% DMSO and stored at -20 ℃; Dulbecco's Modified Eagle's Medium) (DMEM; Gibco Service Co., United States); fetal bovine serum (FBS) from Tianjin Hao Yang Biological Manufacture Co. Ltd. (China); 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Amresco (United States); Hoechst 33258 from Beyotime Institute of Biotechnology (China); Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Assay kit from Beijing Biosea Biotechnology Co. Ltd. (China); lactate dehydrogenase (LDH) assay kit from Nanjing Jiancheng Bioengineering Institute (China).

Cell culture

Cells were cultured in phenol-red-free DMEM containing 10% FBS at a density of 10^5 cells/mL, and placed in a humidified incubator with 95% air and 5% CO_2 at 37 ℃ until cells reached 70%-80% confluence.

Experimental protocol

The complete medium was replaced with DMEM containing 1% FBS 1 d before the experiment for cell synchronization. The cells were randomized into five groups and treated as follows. Cells in the normal control group (N) were kept in normoxic culture for 6 h. In the H/R group, DMEM was replaced with Krebs-Ringer bicarbonate buffer prior to hypoxia induction, and the cells

were transferred into a hypoxic incubator in a humidified atmosphere equilibrated with 94% N_2 + 1% O_2 + 5% CO_2 for 2 h (hypoxia). Afterwards, the Krebs-Ringer bicarbonate buffer was replaced with DMEM for reperfusion simulation, followed by 4 h normoxic culture (for reoxygenation). In the DMSO vehicle postconditioning group (DM), the Krebs-Ringer bicarbonate buffer was replaced with DMEM containing 0.05% DMSO prior to reoxygenation, with no modification of other procedures in the H/R group. In the ghrelin postconditioning group (GH), the Krebs-Ringer bicarbonate buffer was replaced with DMEM containing 10^{-9}, 10^{-8} and 10^{-7} mol/L ghrelin before reoxygenation, with no modification of other procedures in the H/R group. In the D-Lys3-GHRP-6 + ghrelin postconditioning group (D + GH), capsazepine + ghrelin postconditioning group (C + GH) and LY294002 + ghrelin postconditioning group (L + GH), the DMEM was replaced with Krebs-Ringer bicarbonate buffer containing D-Lys3-GHRP-6, capsazepine and LY294002, respectively, followed by 2 h hypoxia. Thereafter, the buffer was replaced with DMEM containing 10^{-7} mol/L ghrelin before reoxygenation, with no modification of other procedures in the H/R group.

MTT assay

The cells in logarithmic growth phase were cultured in a 96-well plate at a density of 10^4 cells/well. Following the experiment in each group, 20 μL MTT (5 mg/mL) was added to each well and incubated for 4 h. Then, 200 μL DMSO was added to each well and the plate was vortexed for 10 min at 37 ℃. A 96-well microplate reader (Thermo, United States) was used to determine $A_{490\ nm}$. Viability (%) was determined as (experimental group $A_{490\ nm}$/normal control group $A_{490\ nm}$) × 100%. Each assay was repeated at least three times.

Hoechst 33258 staining assay

Cells were cultured in six-well plates at a density of 104 cells/well and incubated for 48 h. Following each experiment, cells were fixed with paraformaldehyde for 20 min and washed with 0.01 mol/L phosphate buffered solution (PBS) twice for 3 min, followed by Hoechst 33258 (0.5 mL) staining at 37 ℃ in darkness for 15 min, and rewashed with 0.01 mol/L PBS for 2 min to remove excess background stain. Apoptotic cells were observed by fluorescence microscopy, with excitation wavelength at 350 nm and emission wavelength at 460 nm. The photographs were preserved.

Flow cytometric analysis

Cells were plated in 100 mL culture flasks and incubated for 48 h. Following each experiment, cells were collected, washed twice with ice-cold PBS, followed by cell density determination and dilution in 1 × Annexin-binding buffer to 10^6 cells/mL. Cells was suspended in 200 μL binding buffer and 5 μL Annexin V-FITC for 15 min in darkness. Finally, 300 μL binding buffer and 5 μL propidium iodide (PI) were added to each sample. Apoptosis percentage was analyzed by flow cytometry (Becton Dickinson, United States).

LDH assay

The cells were cultured in 96-well plates at a density of 10^4 cells/well. At the end of each experiment, 20 μL supernatant was drawn from each well and transferred into test tubes, followed by determination of LDH release using the LDH assay kit.

Western blotting

Cells were plated in 100 mL cell culture flasks and incubated for 48 h. At the end of each experiment, the cells were washed thrice in ice-cold PBS prior to lysis with lysis buffer containing 150 mL/L protease inhibitor, followed by cell collection and centrifugation at 4 ℃ for 15 min. The resulting protein-containing supernatant was collected for protein quantification or storage at -80 ℃. The protein contents were determined by bicinchoninic acid assay. Protein samples were adjusted to equal concentration and volume by lysis buffer and then mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The protein samples were heated at 100 ℃ for 5 min, and then 100 μg protein was loaded into each well for 12.5% SDS-PAGE, and the targeted protein was transferred onto a nitrocellulose membrane. Immunoblotting was performed with the following antibodies: mouse anti-Bcl-2 (Zhongshan Golden Bridge Biotechnology Co. Ltd., China), mouse anti-Bax (Zhongshan Golden Bridge Biotechnology), mouse anti-β-actin (Zhongshan Golden Bridge Biotechnology), rabbit anti-VR1 (Zhongshan Golden Bridge Biotechnology), rabbit anti-Akt (Zhongshan Golden Bridge Biotechnology), and rabbit anti-glycogen synthase kinase (GSK)-3β (Wuhan Boshide Biotechnology Co. Ltd., China). The secondary antibodies were alkaline phosphatase goat anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology) and alkaline phosphatase horse anti-mouse IgG (Zhongshan Golden Bridge Biotechnology). Protein expression was quantified by Image J software.

Immunocytochemistry assay

Exponentially growing cells were cultured in a 24-well plate. Immunohistochemistry detection reagent and DAB kit (Zhongshan Golden Bridge Biotechnology) were used to examine the expression of VR1, Akt and GSK-3β in human gastric epithelial cells. Following each experiment, cells were washed thrice in PBS and fixed with 4% paraformaldehyde at 4 ℃ for 30 min. Cells were incubated with 0.3% Triton X-100 for 20 min and 3% H_2O_2 for 10 min. Cells were blocked with 10% normal goat serum for 1 h at room temperature. Primary antibodies were added and incubated at 4 ℃ overnight. With cells washed thrice in cold PBS, the secondary antibody was added and incubated at room temperature for 3 h. Finally, cells were washed thrice in 0.01 mol/L PBS, followed by incubation with DAB complexes for 20 min. Cells were observed for

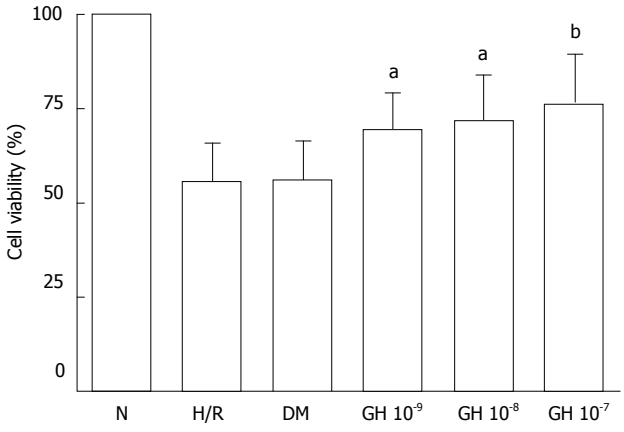


Figure 1 Effects of different doses of ghrelin on cell viability in human gastric epithelial cells induced by H/R. Cells were grouped as follows: normoxic culture for 6 h (N), 2 h hypoxia/4 h reoxygenation (H/R), alcohol vehicle postconditioning (DM) and ghrelin postconditioning at three doses (10^{-9} mol/L, 10^{-8} mol/L and 10^{-7} mol/L). Cell viability was detected by 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. mean ± SD. $n = 6$. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* H/R.

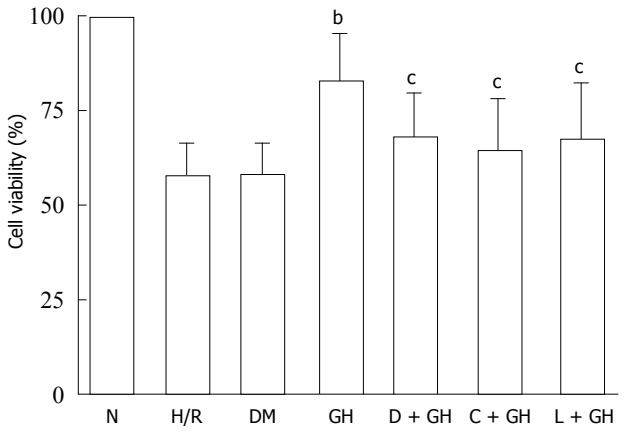


Figure 2 Effects of D-Lys3-GHRP-6, capsazepine and LY294002 in ghrelin postconditioning on cell viability in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (N), 2 h hypoxia/4h reoxygenation (H/R), DMSO vehicle postconditioning (DM), ghrelin postconditioning (10^{-7} mol/L) (GH), D-Lys3-GHRP-6 + ghrelin postconditioning (D + GH), capsazepine + ghrelin postconditioning (C + GH) and LY294002 + ghrelin postconditioning (L + GH). Cell viability was detected by 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. mean ± SD. $n = 6$. $^{b}P < 0.01$ *vs* H/R; $^{c}P < 0.05$ *vs* GH.

photography under a phase contrast microscope.

Statistical analysis

All data were presented as mean ± SD. One-way analysis of variance was performed to determine differences among groups and LSD test was performed to determine differences between groups. All statistical analyses were performed with the SPSS version 13.0 (SPSS, Chicago, IL, United States). The difference was considered statistically significant at $P < 0.05$.

RESULTS

Effects of different doses of ghrelin on cell viability in human gastric epithelial cells induced by H/R

The MTT assay indicated that the GES-1 cells were treated with ghrelin postconditioning at 10^{-9} mol/L, 10^{-8} mol/L and 10^{-7} mol/L. The viability of the GH group was 69.6% ± 9.6%, 71.9% ± 17.4%, and 76.3% ± 13.3%, respectively, in a dose-dependent manner. Compared with the H/R group (55.9% ± 10.0%), the viability significantly increased ($P < 0.05$), suggesting that 10^{-7} mol/L ghrelin was the optimal protective dose, which was used in the subsequent experiments. There were no significant differences between the H/R and DM groups (55.9% ± 10.0% *vs* 56.1% ± 10.5%, $P > 0.05$, Figure 1).

Effects of ghrelin postconditioning on viability of human gastric epithelial cells induced by H/R

To investigate whether GHS-R, VR1 and the PI3K/Akt signaling pathway were related to this effect, their inhibitors D-Lys3-GHRP-6, capsazepine and LY294002 were administered prior to ghrelin postconditioning. The GH group had significantly increased cell viability ($P < 0.01$ *vs* H/R group), whereas the D + GH, C + GH and L + GH groups had significantly decreased cell viability ($P < 0.05$ *vs* GH group, Figure 2), which indicated that D-Lys3-GHRP, capsazepine and LY294002 could reverse the protective effect of ghrelin postconditioning on GES-1 cell viability induced by H/R.

Effects of ghrelin postconditioning on apoptosis of human gastric epithelial cells induced by H/R

Hoechst 33258 staining showed that apoptotic cells were apparent in normal gastric epithelial cells. Compared with the H/R group, there were fewer apoptotic cells in the GH group, whereas many apoptotic cells were observed in the D + GH, C + GH and L + GH groups compared with the GH group (Figure 3).

Flow cytometric analysis showed that the percentage of apoptotic cells in the GH group significantly decreased (12.38% ± 1.51% *vs* 6.88% ± 0.87%, $P < 0.01$) compared with the H/R group. Compared with the GH group, the D + GH, C + GH and L + GH groups had a significantly increased percentage of apoptotic cells (11.70% ± 0.88%, 11.93% ± 0.96%, 10.20% ± 1.05% *vs* 6.88% ± 0.87%, $P < 0.05$). There were no significant differences in the percentage of apoptotic cells between the H/R and DM groups (12.38% ± 1.51% *vs* 13.0% ± 1.13%, $P > 0.05$, Figure 4).

Effects of ghrelin postconditioning on LDH release in human gastric epithelial cells induced by H/R

There was a significant decrease in LDH release in the GH group compared with the H/R group (561.58 ± 64.01 U/L *vs* 1062.45 ± 105.29 U/L, $P < 0.01$). There was a significant increase in LDH release in the D + GH, C + GH and L + GH groups compared with the GH group (816.89 ± 94.87 U/L, 870.95 ± 64.06 U/L, 838.62 ± 118.45 U/L *vs* 561.58 ± 64.01 U/L, $P < 0.01$). There were no significant differences in LDH release between the H/R and DM groups (1062.45 ± 105.29 U/L

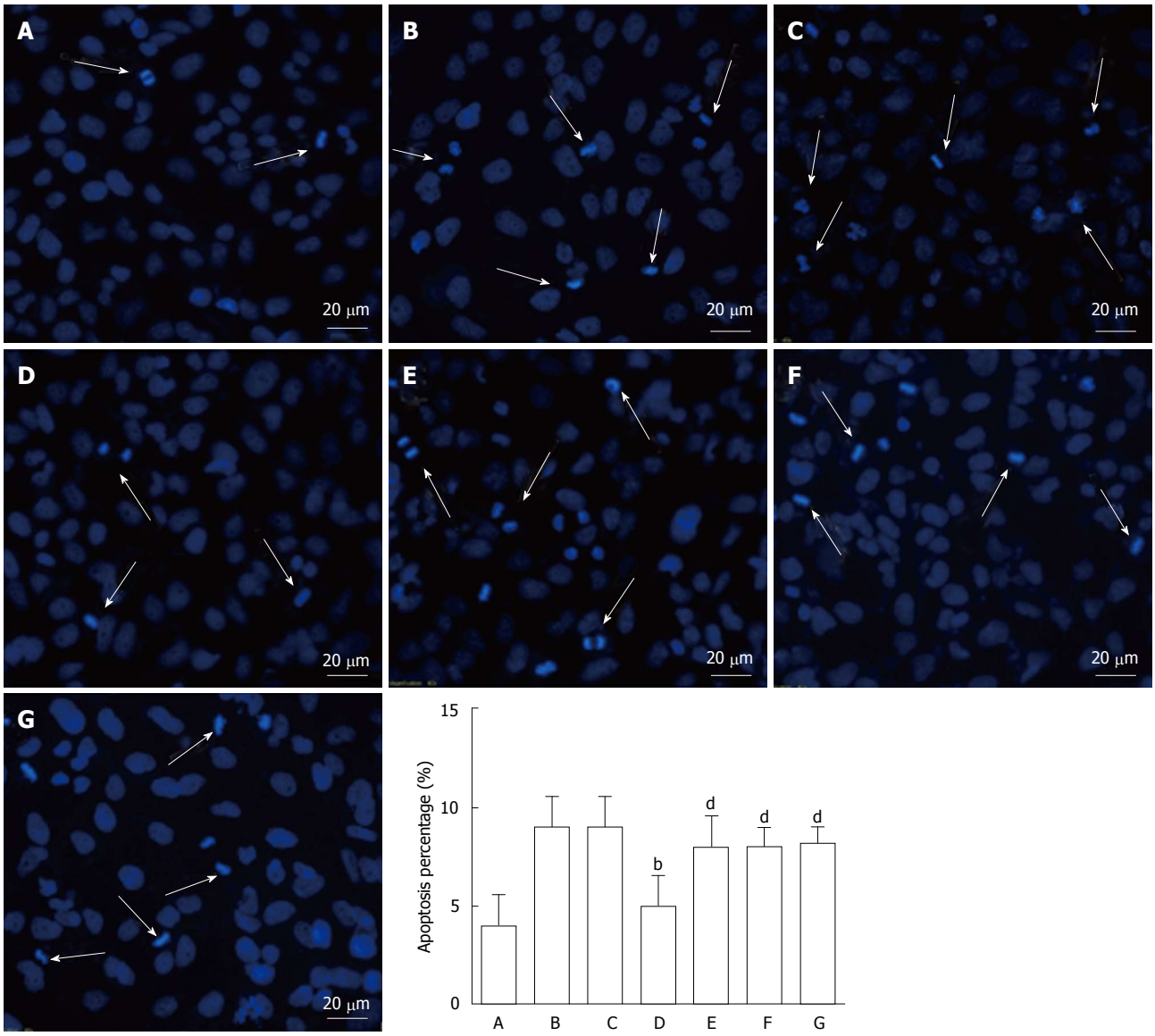


Figure 3 Effects of D-Lys3-GHRP-6, capsazepine and LY294002 in ghrelin postconditioning on cell apoptosis in human gastric epithelial cells induced by H/R. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), D-Lys3-GHRP-6 + ghrelin postconditioning (E), capsazepine + ghrelin postconditioning (F), and LY294002 + ghrelin postconditioning (G). mean ± SD. n = 6. $^{b}P < 0.01$ *vs* B; $^{d}P < 0.01$ *vs* D. Cells were then stained by Hoechst33258. The arrows indicate apoptotic cells.

Table 1 Effects of ghrelin postconditioning on lactate dehydrogenate release

Groups	LDH release (U/L)
N group	672.73 ± 64.79
H/R group	1062.45 ± 105.29
DM group	1017.65 ± 68.90
GH group	561.58 ± 64.01[d]
D + GH group	816.89 ± 94.87[b]
C + GH group	870.95 ± 64.06[b]
L + GH group	838.62 ± 118.45[b]

$^{b}P < 0.01$ *vs* GH group; $^{d}P < 0.01$ *vs* H/R group. N: Normal control group; H/R: Hypoxia/reoxygenation; DM: DMSO postconditioning; GH: Ghrelin postconditioning (10^{-7} mol/L); D + GH: D-Lys3-GHRP-6 + ghrelin postconditioning; C + GH: Capsazepine + ghrelin postconditioning; L + GH: LY294002 + ghrelin postconditioning; LDH: Lactate dehydrogenate.

vs 1017.65 ± 68.90 U/L, $P > 0.05$, Table 1).

Effects of ghrelin postconditioning on expression of Bcl-2, Bax, VR1, Akt and GSK-3β in human gastric epithelial cells induced by H/R

Western blotting demonstrated that Bcl-2, Bax, Akt and GSK-3β were expressed in normal gastric epithelial cells. Compared with the H/R group, expression of Bcl-2 and Akt increased in the GH group, whereas there was a decrease in expression of Bax and GSK-3β ($P < 0.01$). Compared with the GH group, Bcl-2 expression decreased and Bax expression increased in the D + GH, C + GH and L + GH groups ($P < 0.01$). Expression of Akt decreased and expression of GSK-3β increased in the L + GH group ($P < 0.01$, Figure 5).

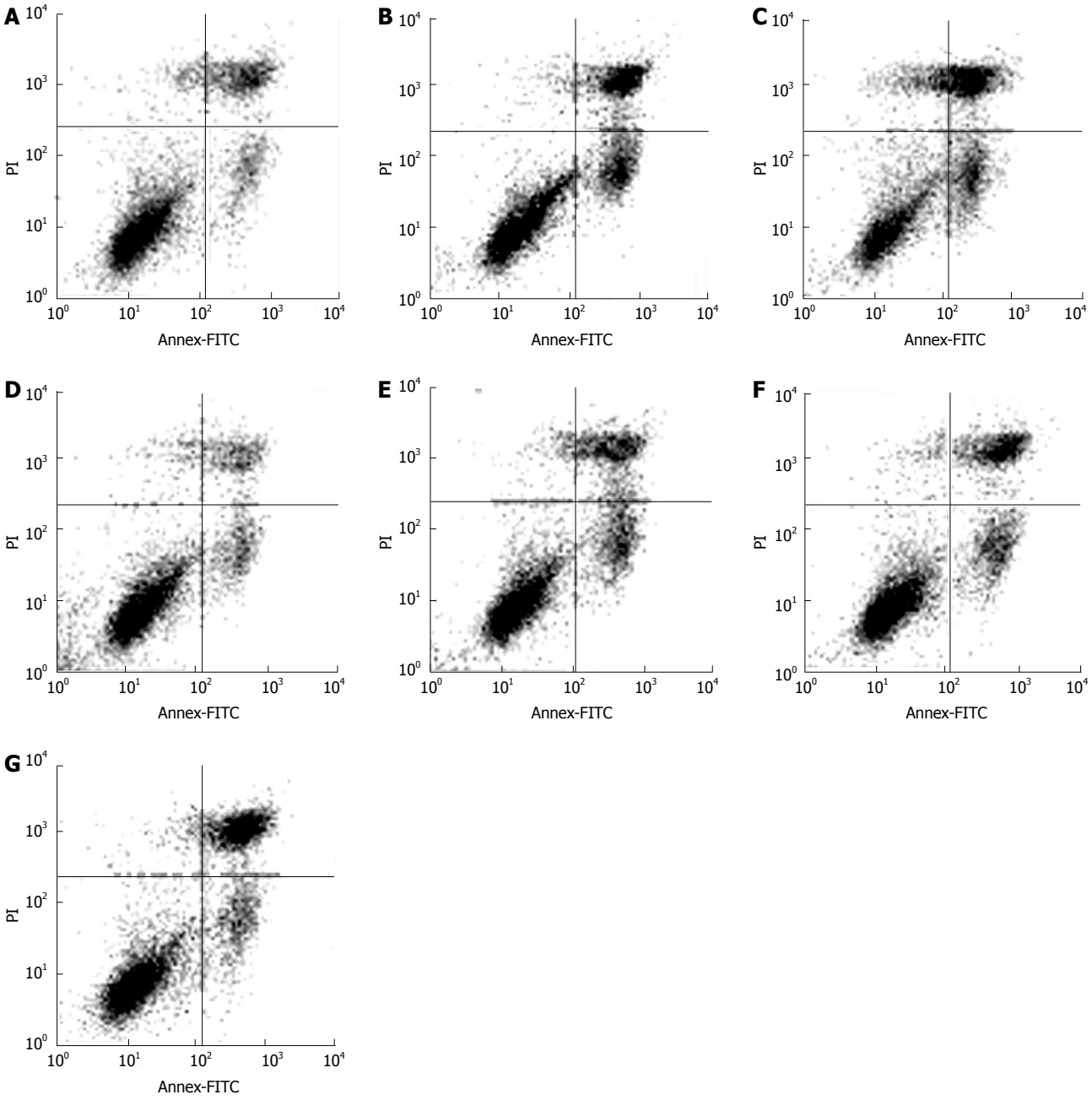


Figure 4 Effects of D-Lys3-GHRP-6, capsazepine and LY294002 in ghrelin postconditioning on cell apoptosis in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), D-Lys3-GHRP-6 + ghrelin postconditioning (E), capsazepine + ghrelin postconditioning (F), and LY294002 + ghrelin postconditioning (G). Subsequently, the cells were stained by annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Apoptosis was detected by flow cytometry.

Immunocytochemistry demonstrated expression of VR1 (Figure 6A), Akt (Figure 7A) and GSK-3β (Figure 8A) in normal human gastric epithelial cells. Two hours hypoxia followed by 4 h incubation under normoxic conditions also upregulated expression of VR1 (Figure 6B) and GSK-3β (Figure 8B) and downregulated Akt (Figure 7B). There was a significant increase in the number of VR1-positive (Figure 6D) as well as Akt-positive (Figure 7D) cells in the GH group, whereas the number of GSK-3β-positive cells significantly decreased (Figure 8D). However, these effects of ghrelin were reversed by capsazepine and LY294002 (Figures 6E, 7E and 8E).

DISCUSSION

Gastric mucosal lesions are frequently observed in clinical situations, such as stress-induced GI/R injury, a major cause of acute gastric mucosal lesions[2,24]. GI/R injury is a common clinical pathophysiological process, which is associated with such factors as excessive generation of oxygen free radicals (OFRs) in gastric mucosa, intracellular calcium overload, increased gastric acid secretion and gastric microcirculation disturbance[25]. Excessive generation of OFRs and intracellular calcium overload may lead to apoptosis[26]. In recent years, there

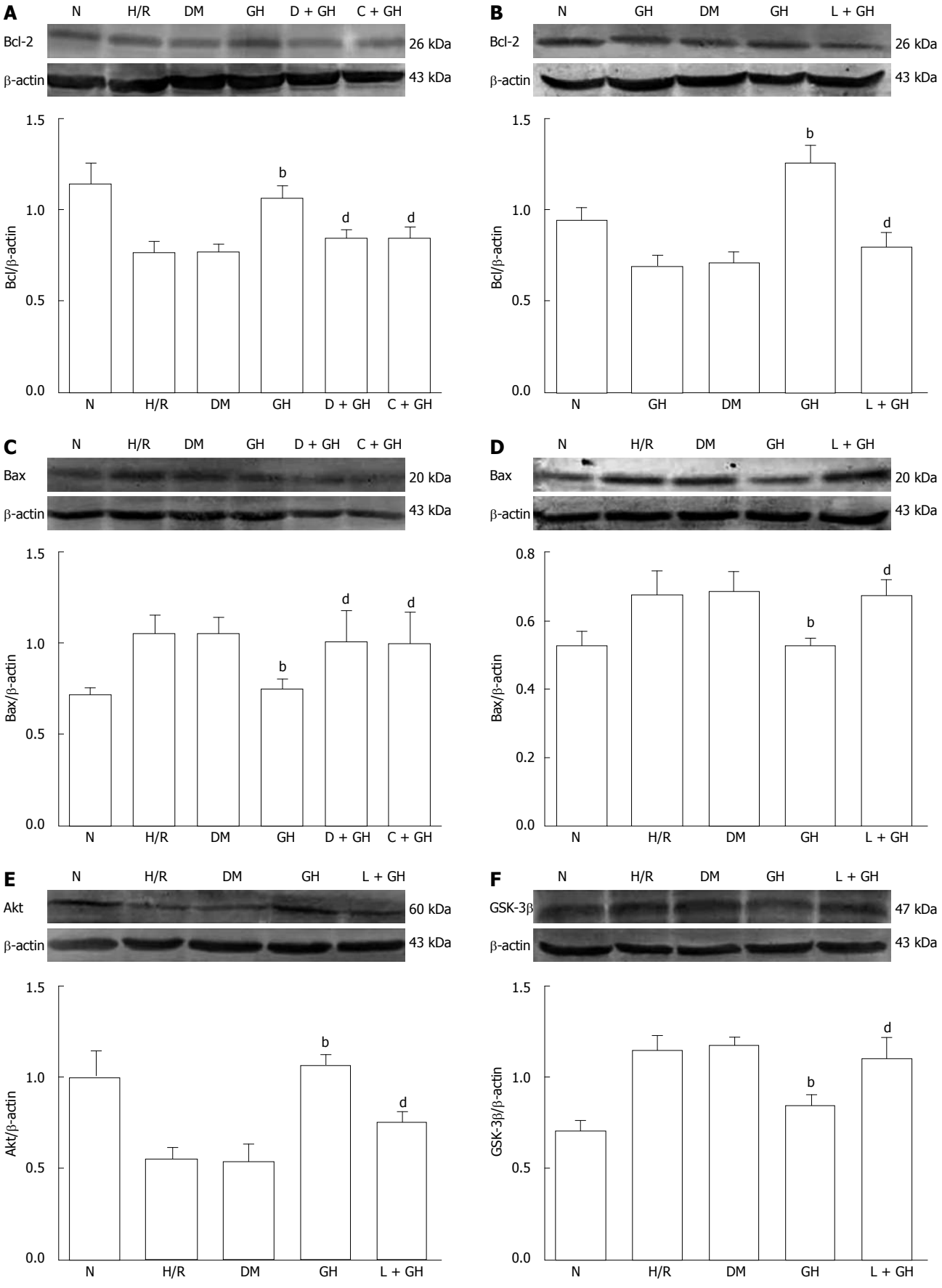


Figure 5 Effects of D-Lys3-GHRP-6, capsazepine and LY294002 in ghrelin postconditioning on the expression of Bcl-2, Bax, Akt and glycogen synthase kinase-3β in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (N), 2 h hypoxia/4 h reoxygenation (H/R), DMSO vehicle postconditioning (DM), ghrelin postconditioning (10^{-7} mol/L) (GH) D-Lys3-GHRP-6 + ghrelin postconditioning (D + GH), capsazepine + ghrelin postconditioning (C + GH) and LY294002 + ghrelin postconditioning (L + GH). The expression of β-actin was detected as an internal standard. Densitometry results are expressed as ratio of test over normal group. A, B: Bcl-2 expression; C, D: Bax expression; E: Akt expression; F: Glycogen synthase kinase (GSK)-3β expression. mean ± SD. $n = 6$. $^{b}P < 0.01$ *vs* H/R, $^{d}P < 0.01$ *vs* GH.

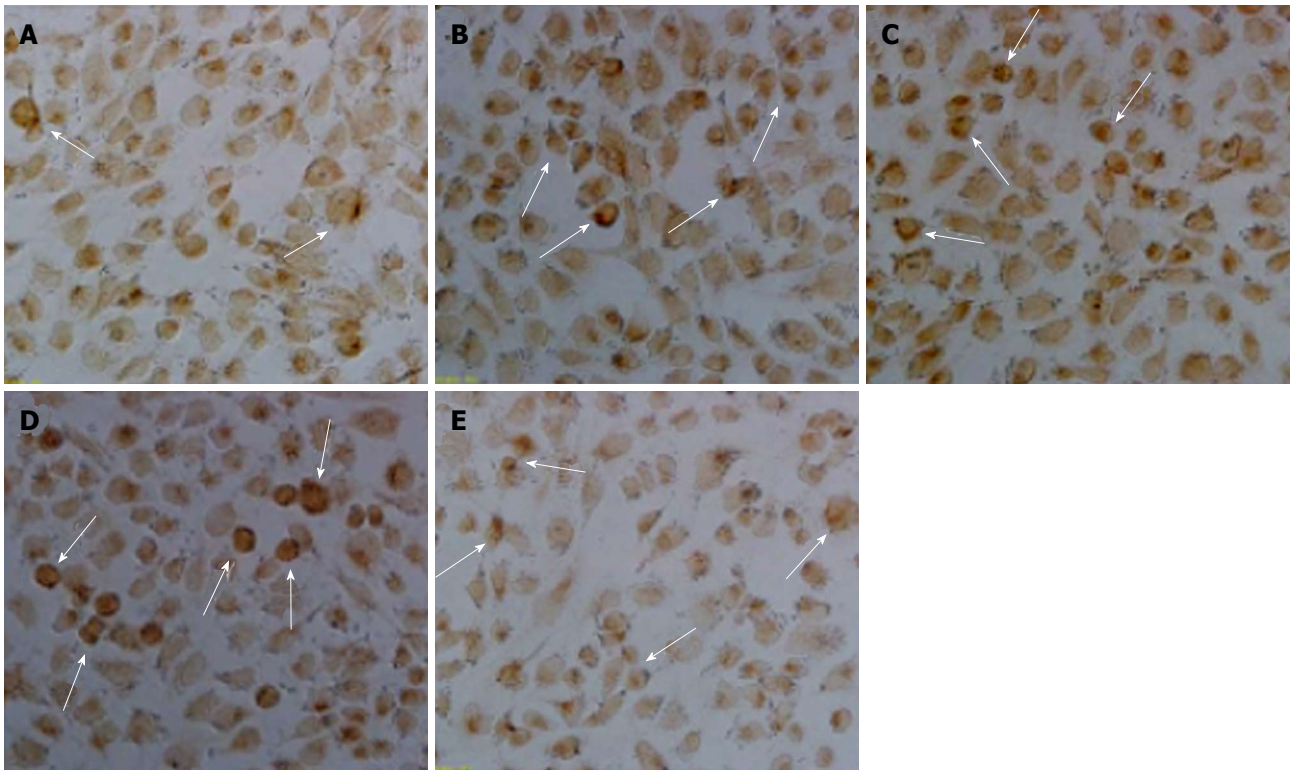


Figure 6 Effects of capsazepine in ghrelin postconditioning on the expression of vanilloid receptor subtype 1 in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), and capsazepine + ghrelin postconditioning (E). The expression of vanilloid receptor subtype 1 (VR1) in each group was observed by immunocytochemistry. Cells were observed for photography under a phase contrast microscope (× 400). The arrows indicate the positive cells which express VR1.

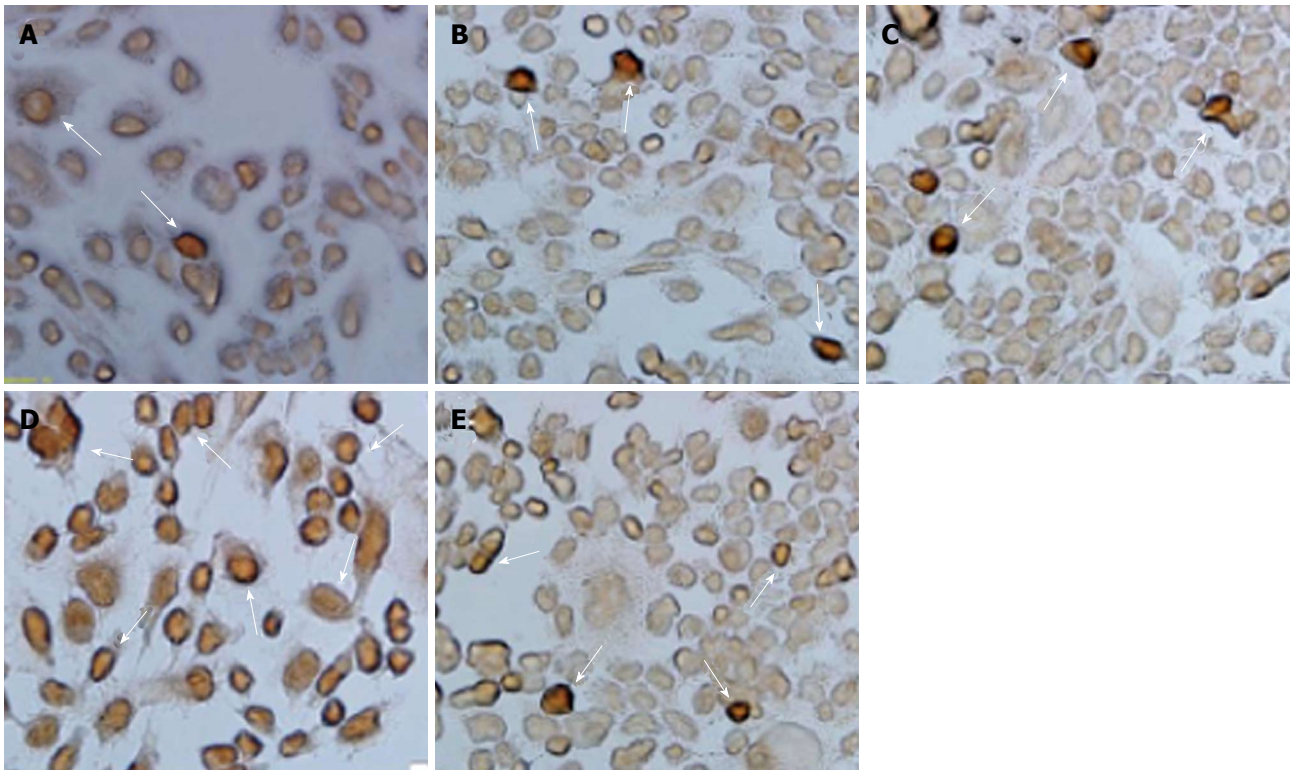


Figure 7 Effects of LY294002 in ghrelin postconditioning on the expression of Akt in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), and LY294002 + ghrelin postconditioning (E). The expression of Akt in each group was observed by immunocytochemistry. Cells were observed for photography under a phase contrast microscope (× 400). The arrows indicate the positive cells which express Akt.

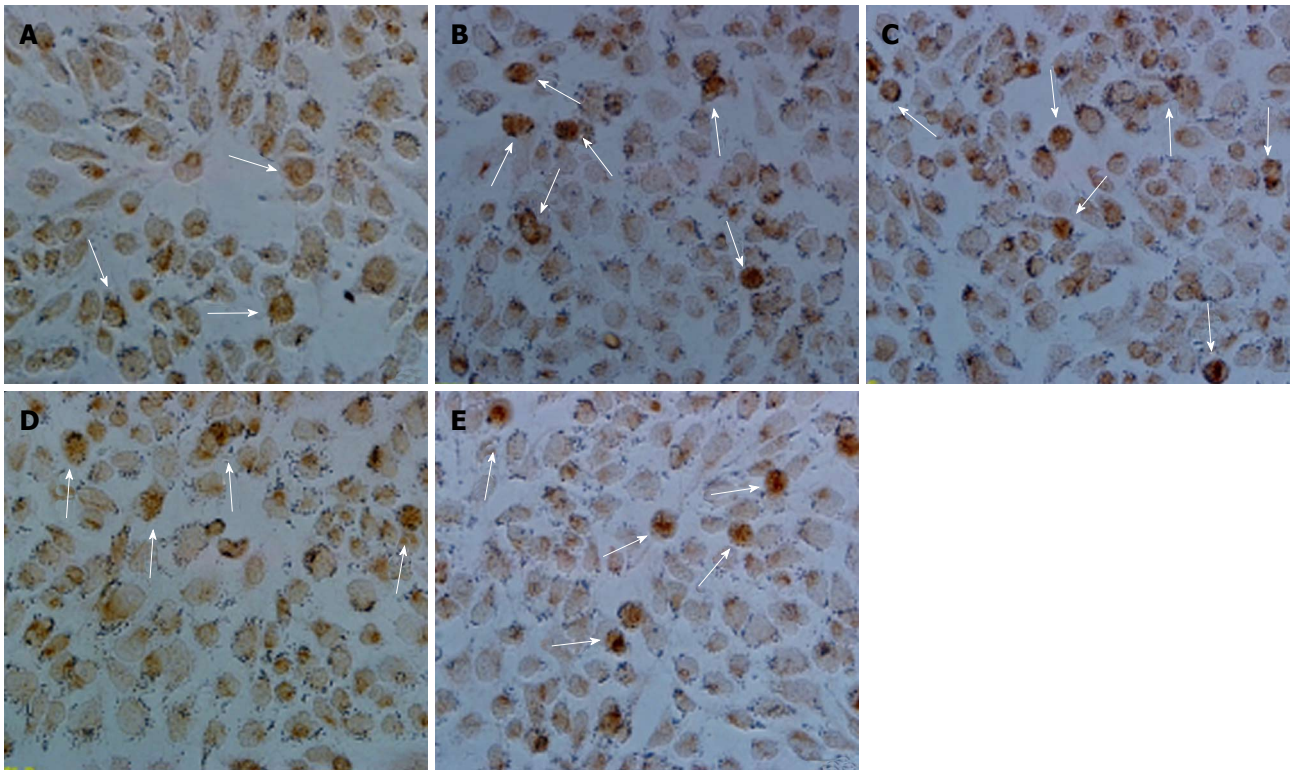


Figure 8 Effects of LY294002 in ghrelin postconditioning on the expression of glycogen synthase kinase-3β in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), and LY294002 + ghrelin postconditioning (E). Expression of glycogen synthase kinase (GSK)-3β in each group was observed by immunocytochemistry. Cells were observed for photography under a phase contrast microscope (× 400). The arrows indicate the positive cells which express GSK-3β.

has been a significant rise in the gastric mucosa cell apoptosis in gastric mucosa injury due to alcohol and nonsteroidal anti-inflammatory drugs, suggesting that there is a process of programmed cell death in acute gastric mucosal injury[27].

Apoptosis is the main mechanism of cell death, and is mediated by a cell-intrinsic suicide program, with the relative balance of pro- and antiapoptotic signaling pathways determining the fate of the cell. There are two main pathways in mammals, one of which is the mitochondrial signaling pathway, in which Bcl-2 plays an important role and Bcl-2/Bax is the key point of apoptosis[28]. In our study, the cell viability significantly decreased and apoptosis percentage increased in the model of 2 h hypoxia/4 h reoxygenation compared with normal control group. They also show that, in the same model, the expression of antiapoptotic protein (Bcl-2) decreased and the expression of proapoptotic protein (Bax) increased. These results suggest that we have successfully established a model of H/R-induced injury in a human GES-1. MTT assays, flow cytometric analysis, LDH assays and western blotting were used to test cell viability, apoptotic percentages, cell LDH release, and apoptosis-related protein expression.

Ghrelin is well known as a potent activator of growth hormone release[8,9]. Given the implication of growth hormone in the tissue regeneration and maintenance of integrity, ghrelin is supposed to contribute to the processes of healing and regeneration. A number of studies have confirmed the effect of ghrelin in gastroprotection. Brzozowski *et al*[17] have found that ghrelin inhibits stress-induced gastric injury[15]. Ghrelin can also inhibit I/R injury. Sibilia *et al*[16] have reported that ghrelin inhibits ethanol-induced gastric ulcers. Unfortunately, all of these were studies *in vivo*. In recent years, many studies have demonstrated that ghrelin confers protection in some cell types *in vitro*, but no study has clearly elucidated the effects of ghrelin in human gastric epithelial cells[29,30]. Therefore, in our study, we demonstrated that ghrelin post-conditioning has protective effects against H/R injury in human gastric epithelial cells, and confirmed that ghrelin could increase cell viability, and decrease apoptosis and LDH release. We also found that ghrelin post-conditioning increased VR1, Akt and Bcl-2 expression, and attenuated GSK-3β and Bax expression subsequent to H/R in gastric epithelial cells *in vitro*. The results showed that ghrelin seems to be involved in the regulation of gastroprotection, which also supports our hypothesis that ghrelin could effectively attenuate H/R-induced injury via the mitochondrial antiapoptotic pathway in human gastric epithelial cells.

In our study, we also demonstrated that the protective effects of ghrelin against H/R-induced injury might be mediated by GHS-R, VR1 and activation of the PI3K/Akt pathway, in that the protective effects of ghrelin were reversed by the GHS-R antagonist D-Lys3-GHRP-6, VR1 antagonist capsazepine and PI3K/Akt antagonist LY294002 during H/R. The result showed that ghrelin in combination with D-Lys3-GHRP-6, capsazepine and

LY294002 decreased cell viability, and increased the percentage of apoptotic cells and LDH release compared with ghrelin post-conditioning. The result also showed a decrease in Bcl-2 expression and increase in Bax expression. In the C + GH group, expression of VR1 was downregulated, and in the L + GH group, expression of Akt decreased, and GSK-3β expression increased. These data suggested that GHS-R, VR1 and the PI3K/Akt signaling pathway might be involved in ghrelin-induced antiapoptotic effects. However, the precise underlying mechanism of their activation by ghrelin remains to be determined.

Ishii *et al*[31] showed that treatment with GHS-R antagonist, D-Lys3-GHRP-6 could partially reverse diabetic hyperphagia. GHS-R mRNA is mainly expressed in the arcuate nucleus of the brain, ventral median nucleus and the hippocampus. It has been demonstrated that GHS-R mRNA is also expressed in peripheral organs[32]. The multifunction of ghrelin coincides with the distribution of GHS-R in various tissues[33]. GHS-R acts as the receptor for a family of synthetic ligands known as growth hormone secretagogues. Therefore, GHS-R is involved in the regulation of physiological actions when activated by its ligands, such as ghrelin. On the grounds of its distribution in the gastrointestinal tract, GHS-R plays an important role in the regulation of gastrointestinal functions[10]. Some studies have shown that GHS-R mediates ghrelin gastroprotection against I/R-induced injury, while this protective effect is inhibited by the GHS-R antagonist D-Lys3-GHRP-6[17].

Capsazepine is a specific and competitive antagonist of the VR. VR1 is a nervous-system-specific receptor. However, recent studies have indicated that VR1 is also distributed in some non-nervous tissues, such as the liver, gastric epithelial cells, bronchi, and bladder epithelium[34-37]. These findings suggest that VR1 might be related to the regulation of a variety of physiological functions of different tissues and organs. Many studies have demonstrated that activation of VR1 by its agonists exerts a gastroprotective effect[35,38-40].

PI3K antagonists, such as LY294002, at low concentrations are considered to be selective PI3K inhibitors and valuable tools for the study of cardioprotection[41]. Many studies have discovered that the activation of the PI3K/Akt signaling pathway is involved in the antiapoptotic effect of ghrelin in many cell types[42-44]. Some studies have shown that the PI3K/Akt pathway is a significant antiapoptotic factor that has an important protective role in reducing the apoptosis-associated protein kinase caspase family, Bcl-2 and other apoptotic factors[23]. The PI3K/Akt pathway has been implicated in the control of major cellular responses including cell proliferation, survival, development, differentiation, cell cycle, and apoptosis[21,22]. GSK-3β is a multifunctional Ser/Thr kinase that plays important roles in necrosis and apoptosis of cardiomyocytes. GSK-3β activity has been associated with many cell processes, including the regulation of multiple transcription factors, the Wnt pathway, nuclear factor κB, endoplasmic reticulum stress, embryogenesis, apoptosis and cell survival, cell cycle progression, and cell migration[45,46]. GSK-3β, serving as an Akt downstream effector, plays an important role during I/R-induced apoptosis of cells in the heart and brain[47,48].

In summary, our study demonstrated that ghrelin postconditioning had a protective effect against H/R-induced injury in human gastric epithelial cells *in vitro*, and the effect might be mediated by the receptors GHS-R and VR1 as well as activation of the PI3K/Akt signaling pathway, resulting in activation of the intracellular antiapoptotic signaling pathway, hence the inhibition of apoptosis.

COMMENTS

Background

Gastric ischemia-reperfusion injury is a common clinical pathophysiological process, and the mobilization of the endogenous protection is the most effective manner against ischemia-reperfusion (I/R) injury. In recent years, ischemic postconditioning has been regarded as important endogenous protection.

Research frontiers

Ghrelin is a 28-amino-acid peptide that was initially identified in rat stomach. Several studies have shown the effect of ghrelin in gastroprotection. In recent years, many studies have demonstrated that ghrelin confers protection in some cell types *in vitro*. Unfortunately, no study has clearly shown the effect of ghrelin in human gastric epithelial cells. In this study, the authors demonstrated that ghrelin post-conditioning has protective effect against hypoxia/reoxygenation (H/R)-induced injury in human gastric epithelial cells.

Innovations and breakthroughs

The injury of visceral I/R is a common pathological occurrence. Nowadays, ischemic preconditioning and ischemic postconditioning have been confirmed to be important approaches in endogenous protection. The notion of ischemic postconditioning was first proposed in 2003. Pharmacological postconditioning is the extension of ischemic postconditioning in which a drug is applied to the ischemic myocardium or hypoxic cardiomyocytes during the initial few minutes of reperfusion or reoxygenation, significantly reducing organ reperfusion injury. This study suggests that the authors have successfully established a model of H/R-induced injury in the human gastric epithelial cell line gastric epithelial cell line (GES-1), and have demonstrated that ghrelin postconditioning has protection against H/R-induced injury in human gastric epithelial cells *in vitro*.

Applications

The results of the study suggest that the ghrelin postconditioning is a potential therapeutic approach that could be applied to the prevention of gastric mucosal lesions induced by ethanol, stress and I/R.

Terminology

Ghrelin, a 28-amino-acid peptide initially identified in rat stomach, is noted for its potent activation of growth hormone release. Pharmacological postconditioning is the extension of ischemic postconditioning in which a drug is applied to the ischemic myocardium or hypoxic cardiomyocytes during the initial few minutes of reperfusion or reoxygenation, significantly reducing organ reperfusion injury.

Peer review

The present study was well-organized and well-investigated. The authors demonstrated the antiapoptotic effects of ghrelin against H/R-induced apoptosis of human gastric epithelial cells.

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BRIEF ARTICLE

Carbon dioxide accumulation during analgosedated colonoscopy: Comparison of propofol and midazolam

Ludwig T Heuss, Shajan Peter Sugandha, Christoph Beglinger

Ludwig T Heuss, Shajan Peter Sugandha, Christoph Beglinger, Department of Gastroenterology, University Hospital, 4031 Basel, Switzerland
Author contributions: Heuss LT and Beglinger C designed the study; Heuss LT and Sugandha SP contributed to data collection and data analysis; Heuss LT, Sugandha SP and Beglinger C wrote the paper.
Correspondence to: Ludwig T Heuss, MD, MBA, Department of Gastroenterology, University Hospital, 4031 Basel, Switzerland. ltheuss@hin.ch
Telephone: +41-44-3972012 Fax: +41-44-3972688
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Abstract

AIM: To characterize the profiles of alveolar hypoventilation during colonoscopies performed under sedoanalgesia with a combination of alfentanil and either midazolam or propofol.

METHODS: Consecutive patients undergoing routine colonoscopy were randomly assigned to sedation with either propofol or midazolam in an open-labeled design using a titration scheme. All patients received 4 μg/kg per body weight alfentanil for analgesia and 3 L of supplemental oxygen. Oxygen saturation (SpO_2) was measured by pulse oximetry (POX), and capnography ($PcCO_2$) was continuously measured using a combined dedicated sensor at the ear lobe. Instances of apnea resulting in measures such as stimulation of the patient, a chin lift, a mask maneuver, or withholding of sedation were recorded. $PcCO_2$ values (as a parameter of sedation-induced hypoventilation) were compared between groups at the following distinct time points: baseline, maximal rise, termination of the procedure and 5 min after termination of the procedure. The number of patients in both study groups who regained baseline $PcCO_2$ values (± 1.5 mmHg) five minutes after the procedure was determined.

RESULTS: A total of 97 patients entered this study. The data from 14 patients were subsequently excluded for clinical procedure-related reasons or for technical problems. Therefore, 83 patients (mean age 62 ± 13 years) were successfully randomized to receive propofol (n = 42) or midazolam (n = 41) for sedation. Most of the patients were classified as American Society of Anesthesiologists (ASA) Ⅱ [16 (38%) in the midazolam group and 15 (32%) in the propofol group] and ASA Ⅲ [14 (33%) and 13 (32%) in the midazolam and propofol groups, respectively]. A mean dose of 5 (4-7) mg of Ⅳ midazolam and 131 (70-260) mg of Ⅳ propofol was used during the procedure in the corresponding study arms. The mean SpO_2 at baseline (%) was 99 ± 1 for the midazolam group and 99 ± 1 for the propofol group. No cases of hypoxemia (SpO_2 < 85%) or apnea were recorded. However, an increase in $PcCO_2$ that indicated alveolar hypoventilation occurred in both groups after administration of the first drug and was not detected with pulse oximetry alone. The mean interval between the initiation of sedation and the time when the $PcCO_2$ value increased to more than 2 mmHg was 2.8 ± 1.3 min for midazolam and 2.8 ± 1.1 min for propofol. The mean maximal rise was similar for both drugs: 8.6 ± 3.7 mmHg for midazolam and 7.4 ± 3.2 mmHg for propofol. Five minutes after the end of the procedure, the mean difference from the baseline values was significantly lower for the propofol treatment compared with midazolam (0.9 ± 3.0 mmHg *vs* 4.3 ± 3.7 mmHg, P = 0.0000169), and significantly more patients in the propofol group had regained their baseline value ± 1.5 mmHg (32 of 41 *vs* 12 of 42, P = 0.0004).

CONCLUSION: A significantly higher number of patients sedated with propofol had normalized $PcCO_2$ values five minutes after sedation when compared with patients sedated with midazolam.

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Key words: Colonoscopy; Deep sedation; Propofol; Hypoventilation; Blood gas monitoring; Transcutaneous

Peer reviewer: Spiros Ladas, Professor, 1st Department of Internal Medicine-Propaedeutic, Medical School, Athens University, "Laiko" General Hospital, Agiou Thoma 17, Athens 11527, Greece

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INTRODUCTION

Colonoscopies are usually performed under sedation with an intravenous sedative that is often combined with an analgesic drug[1,2]. A combination of the benzodiazepine midazolam with an opioid is the most commonly used regimen in Western countries[3]. During the last few years, propofol (2,6-diisopropylphenol) sedation has gained increasing attention among endoscopists as an alternative sedative in GI endoscopy[4-6]. With a fast onset of action of 30-60 s, a distribution half-life of 2-4 min, and a rapid recovery time, propofol combines the major characteristics of an ideal sedative[7].

In recent years, several randomized trials have compared midazolam *vs* propofol with regard to patient safety and satisfaction[4,8-10]. In most studies, recovery time, measured as completely regained alertness after the endoscopic procedure, was used as the main endpoint. Although the advantages of propofol regarding these pharmacokinetic properties are unquestionable, there is still an ongoing debate on the potential respiratory impairment hazards of propofol when used by non-anesthesiologists. In contrast to benzodiazepines, a reversal agent for propofol does not exist; consequently, the use of propofol requires special attention. Furthermore, a deeper level of sedation may be reached with propofol that carries the risk of unintentional deep sedation or even general anesthesia[11,12].

Standard monitoring with pulse oximetry (POX) may miss hypoventilation, which is much better reflected by an increase in arterial carbon dioxide tension[13,14]. Arterial blood gas analysis is the 'gold standard' method to measure the arterial partial pressure of carbon dioxide ($PaCO_2$). However, arterial sampling, including arterial catheterization, is invasive and expensive. Transcutaneous carbon dioxide tension ($PcCO_2$) measurement is used as a noninvasive surrogate measure of $PaCO_2$ and to estimate $PaCO_2$ or determine trend changes in the measurement. Recently, considerable progress has been made in the technical aspects of $PcCO_2$ monitoring. A single earlobe sensor can now measure $PcCO_2$ and pulse oximetry simultaneously. Transcutaneous carbon dioxide tension measurement has been shown to be a reliable monitoring technique that corresponds well with $PaCO_2$ values measured in arterial blood gas samples[15-17].

The aim of this study was to evaluate the profile of $PcCO_2$ as a marker of hypoventilation during sedation with propofol or midazolam in colonoscopies.

MATERIALS AND METHODS

This was an open-labeled, blinded, randomized prospective study. Consecutive outpatients undergoing elective colonoscopy and opting for sedation were randomly assigned to receive propofol or midazolam. The patients were assigned using randomly numbered opaque envelopes. As a standard procedure, all patients received 3 L/min supplemental oxygen and analgesia with 4 μg/kg per body weight (BW) alfentanil (Rapifen®, Janssen-Cilag, Baar, Switzerland) prior to sedation[18]. The first bolus of the sedative drug was administered exactly one minute after the alfentanil. Midazolam (Dormicum®, Hoffmann La Roche AG, Basel, Switzerland) was administered in a first dose of 2 mg; further boluses of 1 mg were administered with an interval of at least 1 min or more depending on the clinical outcome. Propofol (Disoprivan®, AstraZeneca, Zug, Switzerland) was administered in two boluses of 20 mg followed by further boluses of 10 mg after an interval of at least 20 s. The sedative drugs were administered by registered nurses under the supervision of the endoscopist based on the clinical response of the patient; the nurses followed our institutional protocol as published elsewhere (nurse-administered propofol sedation or NAPS)[18]. The nurse administering the sedation had no tasks except to monitor the patient and administer sedation. A different nurse assisted the endoscopist with the technical performance of the procedure. Monitoring consisted of the measurement of continuous oxygen saturation, electrocardiography and heart rate, as well as regular measurements of blood pressure. The primary method of monitoring was the nurse's clinical assessment of the patient, including measurement of respiratory effort by visual assessment and by palpation of the chest wall and abdominal excursion and/or palpation of exhaled breath.

A short personal history was obtained from all the patients, and their general physical condition was assessed using the American Society of Anesthesiologists (ASA) classification. Exclusion criteria were as follows: (1) a known history of intolerance to propofol (including sensitivity to eggs and soybeans); (2) an age less than 18 or more than 85 years; (3) an ASA score of Ⅳ or Ⅴ; (4) a known high grade of aortic (gradient > 80 mmHg) or carotid stenosis (> 75%); and (5) intravenous drug abuse.

All endoscopic examinations were performed according to the department's standard operating procedures with different types of Pentax video colonoscopies (Pentax, Hamburg, Germany) using regular room air to insufflate the colon. The colonoscopies were conducted by seven different expert endoscopists, including four experienced gastroenterologists and three residents in their last year of gastroenterology training, who all had performed more than 400 colonoscopies each. The pro-

cedures were performed in an x-ray suite equipped for fluoroscopy (straightening maneuvers and confirming instrument position when necessary). The decision to perform an ileal intubation or an endoscopic intervention (e.g., polypectomy) depended on the clinical situation and was decided by the endoscopist.

The local ethics committee approved the study protocol, and written informed consent was obtained from the patients before study enrollment.

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We used a recently developed combined POX/$PcCO_2$ sensor (V-Sign™, Sentec AG, Therwil, Switzerland) weighing 3 g that was placed at the right earlobe with a dedicated ear clip. We used a recently developed combined POX/$PcCO_2$ sensor (V-Sign™, Sentec AG, Therwil, Switzerland) weighing 3 g that was placed at the right earlobe with a dedicated ear clip[17]. This fully digital sensor combines the elements of an electrochemical Severinghaus-type carbon dioxide tension sensor with those of conventional optical POX sensors, thus providing noninvasive and continuous estimation of $PaCO_2$ and SaO_2[19]. The sensor was warmed to a constant surface temperature of 42 ℃ to improve local arterialization and to accelerate carbon dioxide diffusion. After the sensor was positioned, the SpO_2 values were available immediately, whereas the $PcCO_2$ values required an equilibration time of approximately 4-5 min. The system is designed to be "ready-for-use" by automated recalibration every time the sensor is placed on the docking station between measurements. The system was previously shown to deliver accurate and reproducible results for $PcCO_2$ and POX[17]. The system was also shown to have an excellent correlation between oxygen saturation and carbon dioxide measurements when the combined sensor was compared with arterial blood gas analysis[17,20].

Readings from the POX/$PcCO_2$ sensor (placed at the right ear lobe) were continuously recorded and stored on a personal computer. An independent observer who was blinded to the type of sedation recorded the readings. Similarly, the procedural personnel were blinded to the $PcCO_2$ data. All collected data were visualized using statistic graphics software (Igor Pro 4.01, WaveMetrics Inc., Lake Oswego, OR). Values at defined time points were thereafter identified manually. $PcCO_2$ values (as a parameter of sedation-induced hypoventilation) were compared between the study groups at the following distinct time points: at baseline, at the time point of maximal increase, at the time point when the cecum was reached, at the end of the procedure and 5 min after the end of the procedure.

The primary endpoint was defined as the number of patients in both study groups who regained their baseline $PcCO_2$ value (± 1.5 mmHg) five minutes after the end of the procedure. Secondary end points included the mean time lag between the application of the sedative drug and an increase of the $PcCO_2$ curve of more than 2 mm Hg, safety parameters defined as apnea with the need for intervention (in case of a decrease in SaO_2 values below 85% for more than 20 s), the frequency of SaO_2 decreases below 90% and a decrease of the heart rate below 50 bpm. Apnea was defined as a lack of spontaneous respiratory effort for more than 20 s and was assessed clinically by the nurse administrating the sedation. An increase of $PcCO_2$ above the baseline was defined as hypoventilation. The target sedation level was a quiet patient in both of the groups as implemented using our NAPS protocol, which has been in practice at this center for several years. The recovery time for all of the patients was defined as the time required for completion of the procedure, i.e., a complete withdrawal of the instrument and simultaneous conclusion of all sedation. As a standard of practice, the patients were transferred to a quiet recovery room following the final 5-min recording of the$PcCO_2$ readings in the endoscopy suite.

All of the parameters were analyzed using descriptive statistics (mean, standard deviation). Categorical outcomes were analyzed using the χ^2 or F test as appropriate. Continuous parameters were analyzed using an analysis of variance, and for non-continuous parameters, the Mann-Whitney Test was used. $P < 0.05$ was defined as statistically significant. All tests were two-sided. For all statistical calculations, SPSS software (SPSS for Windows, Version 11.0, SPSS Inc., Chiago, Illinois) was used. The sample size calculation was based on the primary outcome of this study of detecting a $PcCO_2$ difference of < 1.5 mmHg from the baseline at 5 min after the end of the procedure in the propofol group. In a pilot study in a similar population, a difference in values between midazolam and propofol was observed in 4 of 26 patients (15.4%). As we expected a clinical/physiological relevant effect, the sample size was determined to detect an ad-

Table 1 Demographic data of the randomized groups

	Sedation with Midazolam (*n* = 42)	Sedation with Propofol (*n* = 41)	*P* value
Age (yr, mean ± SD)	62 ± 13	62 ± 13	NS
M : F (*n*)	19:23	20:21	NS
ASA Ⅰ	12 (29%)	13 (32%)	NS
ASA Ⅱ	16 (38%)	15 (37%)	NS
ASA Ⅲ	14 (33%)	13 (32%)	NS
Smoker	6 (15%)	6 (15%)	NS
Mean dosage of sedative in mg (range)	5 (4-7)	131 (70-260)	-

ASA: American Society of Anesthesiologists classification; NS: Not significant.

Table 2 Oxygen saturation measured by pulse oximetry at baseline and changes during endoscopy according to sedatives

	Midazolam (*n* = 42)	Propofol (*n* = 41)	*P* value
SpO_2 at baseline (%)	99 ± 1	99 ± 1	NS
Mean max decrease of SpO_2 (%)	6 ± 3	4 ± 2	NS

SpO_2: Oxygen saturation measured by pulse oximetry; NS: Not significant.

Table 3 Procedure outcomes according to the defined endpoints (*n*)

	Midazolam (*n* = 42)	Propofol (*n* = 41)	*P* value
$\Delta PcCO_2$ < ± 1.5 mmHg (from baseline and five min after end)	12	32	0.0004
SpO_2 < 85%	0	0	NS
SpO_2 < 90%	6	0	0.05
HR < 50 bpm	5	1	NS
Decrease MAP > 25%	17	17	NS

HR: Heart rate; MAP: Mean arterial pressure; SpO_2: Oxygen saturation measured by pulse oximetry; $\Delta PcCO_2$: Difference of transcutaneous carbon dioxide tension; NS: Not significant.

ditional increase of 75% in the midazolam group with a power of 80%. Thirty-eight patients in each group would be required to detect such a difference ($P = 0.05$) (nQuery Advisor, Version 5.0, Statistical Solutions, Saugus, MA, United States).

RESULTS

Among the 133 colonoscopies performed during the study period, 97 patients were successfully randomized to the study. Fourteen randomized patients had to be subsequently excluded for the following reasons: in 5 patients, the procedure was not completed because of incomplete bowel preparation; in 5 patients, a short disconnection of the sensor provided no continuous data; in two patients, a calibration fault occurred because of the prototype calibration unit used, and the endoscopist refrained from recalibration; and in two patients, the sedative drug was not correctly administered according to the protocol. Therefore, the final study population contained 83 patients. Demographic characteristics of the study groups are shown in Table 1. The mean duration of the procedures was 26 ± 13 min for the midazolam group and 27 ± 18 min for the propofol group (not significant).

Decreased ventilation activity was detected in all patients to whom a sedative was administered; on average, the $PcCO_2$ values increased by 8.0 ± 3.7 mmHg. The mean SpO_2 values at baseline and the mean maximal decrease during sedation are shown in Table 2.

When comparing midazolam and propofol, different profiles were observed for the $PcCO_2$ readings, as shown in Figure 1. The increase in $PcCO_2$ was mostly related to a short delay in the administration of incremental dosages of the sedatives. The mean time interval after the first application of the drug until the $PcCO_2$ value had increased by more than 2 mmHg was 2.8 ± 1.3 min for midazolam and 2.8 ± 1.1 min for propofol. Although there was no significant difference in the mean increase in $PcCO_2$ following administration of midazolam or propofol, the patients who received midazolam tended to remain in a prolonged state of decreased ventilation when compared with the patients receiving propofol. The difference in $PcCO_2$ values (baseline compared with the end of the procedure) was significantly higher in the patients receiving midazolam. Therefore, the patients who received propofol had a $PcCO_2$ level that was significantly closer to the baseline five minutes after the end of the procedure when compared with the patients who received midazolam (Tables 2, 3, Figure 2).

None of the study patients manifested apnea or hypoxemia below 85%. Drops in oxygen saturation below 90% tended to occur more often during sedation with midazolam than with propofol (Table 3).

DISCUSSION

Achieving higher safety standards for sedation in routine endoscopy has been a priority over the last few years. In keeping with this objective, the present study showed that patients undergoing colonoscopy under sedation developed relative hypoventilation (as reflected by retention of CO_2) that persisted for a significantly longer period in patients sedated with midazolam than in patients sedated with propofol. The results support the findings of a meta-analysis that suggested that propofol sedation during colonoscopy is associated with a lower risk of complications when compared with traditional sedative agents[21].

Although the use of oxygen saturation monitoring during sedation is routinely used by most gastroenterologists and the administration of supplemental oxygen has become a widely accepted practice, little attention has been paid to the development of alveolar hypoventilation[17]. Because of the buildup of CO_2 in the patient's alveoli and blood, hypoventilation can be effectively detected by capnography, which has recently become a focus of interest as an additional monitoring parameter during gastrointestinal procedures. This procedure has become

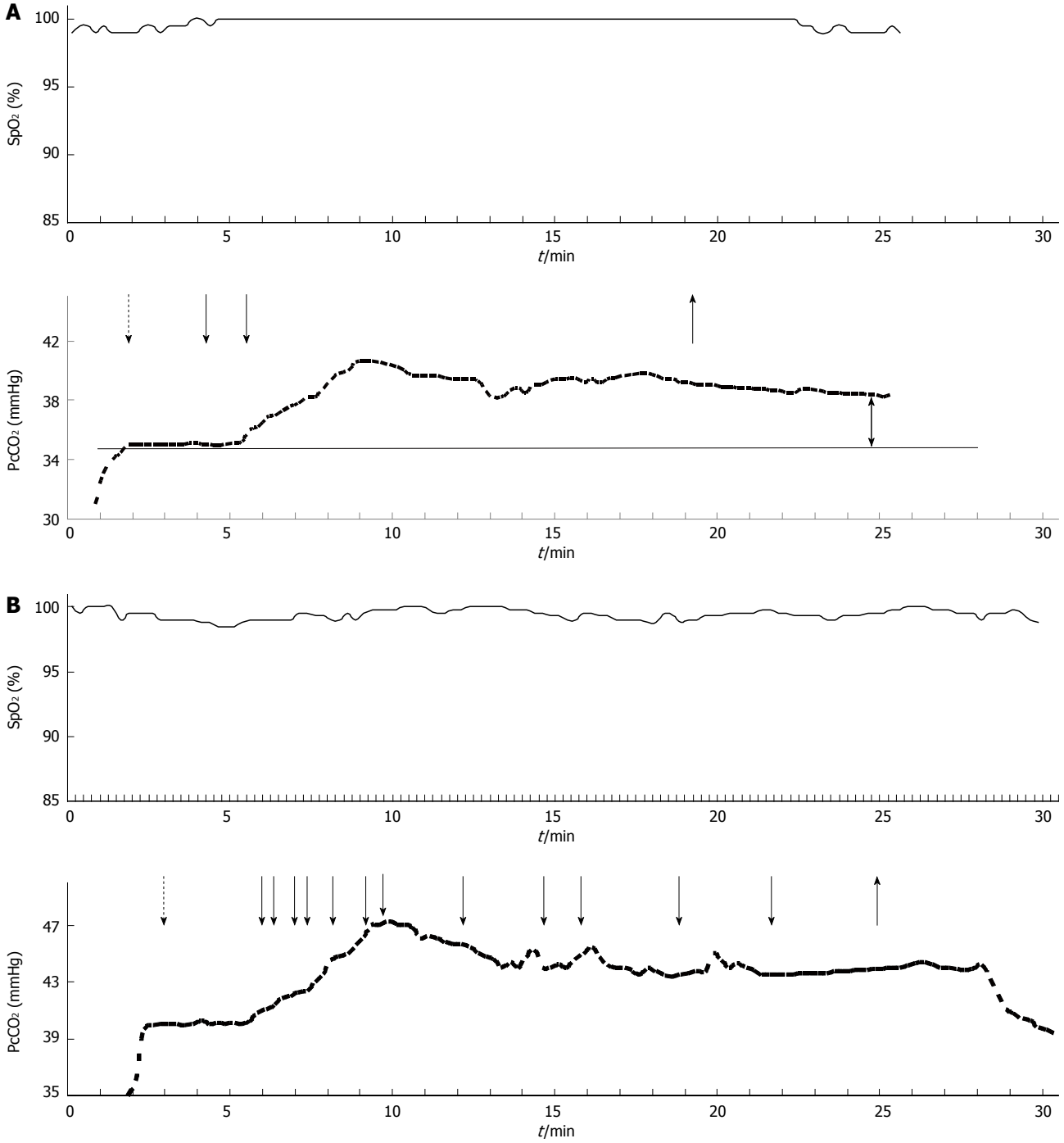


Figure 1 A typical course of oxygen saturation measured by pulse oximetry and transcutaneous carbon dioxide tension following administration of alfentanil (dashed arrow) and midazolam (A) or propofol (B) (solid arrows as indicated). The upright arrow indicates the end of the procedure. The double arrow highlights the difference after termination of the procedure.

relevant as the use of newer anesthetic drugs such as propofol has increased[15].

Several studies to date have evaluated the importance of CO_2 buildup during endoscopic procedures[14-16,22]. Freeman *et al*[15] were the first to show that profound hypoventilation may frequently occur undetected during a gastrointestinal endoscopy, especially if additional oxygen is given and the decline of oxygen saturation is thus prevented; we observed a similar effect in our study. Freeman *et al*[15] also found that the degree of hypoventilation was more closely related to the sedative drug dose than to the underlying medical illness. In 30 colonoscopies, with 90% receiving fentanyl and 37% additionally receiving midazolam, Freeman *et al*[15] recorded a mean PCO_2 increase of 6.4 ± 3.8 mmHg, whereas during ERCP with a higher rate of deeper sedation, the mean values were 14.2 ± 10.2 mmHg. Our results showed a mean increase in $PcCO_2$ of 8.6 ± 3.7 mmHg despite a continuous SpO_2 above 90%; these results are similar to the experience of Freeman *et al*[15].

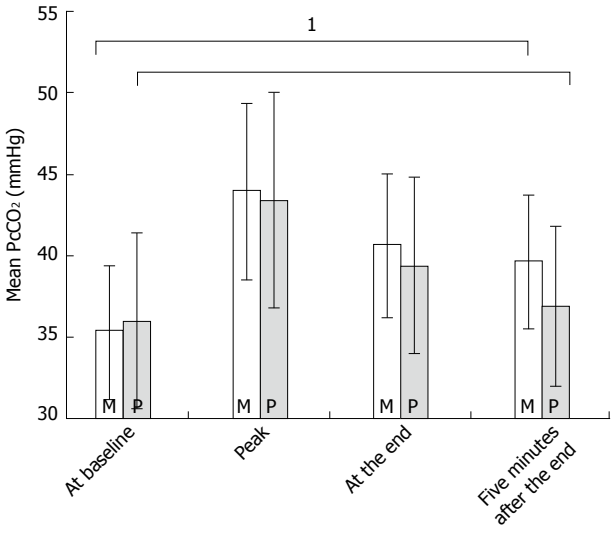


Figure 2 Mean transcutaneous carbon dioxide tension values in mmHg (± SD) at four distinct time points during colonoscopies according to sedative use (for the whole group). [1]A significant difference in the transcutaneous carbon dioxide tension values measured five minutes after the end of the procedure when compared with the baseline values. M: Midazolam; P: Propofol.

The pattern of the registered $PcCO_2$ readings differed noticeably in our study according to the pharmacological properties of the administered drugs. The pattern of the initial rise of $PcCO_2$ was similar, but the $PcCO_2$ level decreased much earlier after the administration of propofol than after the administration of midazolam. Although propofol had to be administered more often in a repeated fashion to maintain the desired sedation level, its effect ceased much faster than the effect of midazolam. This pharmacologic pattern seems to be reflected by the shape of the $PcCO_2$ curve.

We did not observe severe hypoxemia or apnea in either of the study groups; the increase in CO_2 could, however, indicate silent risk during poor sedative practice. In the study by Freeman *et al*[15]. We did not observe severe hypoxemia or apnea in either of the study groups; the increase in CO_2 could, however, indicate silent risk during poor sedative practice. In the study by Freeman *et al*[15], one case demonstrated an increase of the $PcCO_2$ curve above 80 mmHg prior to respiratory arrest. Nelson *et al*[16] showed that the monitoring of $PcCO_2$ may be useful for the endoscopist to guide sedation using midazolam and fentanyl and that this monitoring can help to prevent severe carbon dioxide retention. Thus, we believe that determining the $PcCO_2$ level can be helpful for the endoscopist when deciding whether to administer a further incremental dose of the sedative.

The combination used in this study of alfentanil, which is a potent opioid with a rapid onset of action, and midazolam is uncommon. Typically, this substance alfentanil is administered in combination with propofol for patient-controlled sedation or for short sedoanalgesia in emergency medicine[23]. The rationale to use this substance alfentanil as a single bolus at the beginning of the procedure was (1) to provide the patients with optimal analgesia during the most painful passage of the sigmoid colon; and (2) to determine whether differences in alveolar hypoventilation at the end of the procedure can be completely attributed to the pharmacologic effect of the sedatives. Furthermore, if propofol is used in outpatient procedures, alfentanil may be an ideal drug to use in combination because of its pharmacokinetic properties and analogous profile of action. Because alfentanil (similarly to all opioids) can induce or enhance alveolar hypoventilation, our protocol prescribed a single low dose of 4 μg/kg per BW of alfentanil and a strict time interval of administration one minute before the first titration dose of the sedative was given.

Alveolar hypoventilation exists when the arterial $PaCO_2$ increases above 45 mmHg, which can occur as the result of various underlying factors. Sedation directly or indirectly influences alveolar hypoventilation by a predominant central effect, thereby causing an increase in the $PaCO_2$. Therefore, recognition and adequate monitoring of this physiological change through indirect means such as transcutaneous monitoring of $PcCO_2$ could play an important role during sedation. The peak $PcCO_2$ value may not be clinically relevant; however, the time period during which reduced ventilation occurs may represent a period of increased risk for some patients. Because of the S-shape of the oxygen dissociation-curve, hypoventilation accompanied by a decrease in PaO_2 may remain unnoticed over time. Although a patient would have adequate arterial saturation with the administration of supplemental oxygen, an adverse physiologic trend that may be reflected by changes in the $PaCO_2$ may go unnoticed.

Transcutaneous CO_2 monitoring in adults has yielded conflicting results because of technological limitations, such as the time required for calibration, the need to warm the skin to 42 degrees, the effect of sweating and the influence of skin metabolism and thickness. Technical problems precluded an accurate interpretation of the data in 7 of the 97 patients and thus represent a limitation in our study.

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warm the skin to 42 degrees, the effect of sweating and the influence of skin metabolism and thickness. Technical problems precluded an accurate interpretation of the data in 7 of the 97 patients and thus represent a limitation in our study.

The main focus of this study was to evaluate the impairment of ventilation induced by midazolam or propofol during colonoscopies. Most trials comparing the use of propofol and midazolam in the endoscopy suite have focused on differences in recovery time (assessed using a discharge scoring system, for example)[24]. The present study suggests that there is also a significant difference in the duration of hypoventilation during the post-procedural period. Although alveolar hypoventilation is generally well tolerated by most patients, it may nevertheless be of clinical relevance in patients with compromised health. Iber *et al*[25] showed that in 4% of patients sedated with midazolam, a relevant decrease of oxygen saturation below 89% occurred during the 30 min after the endoscopic procedure, which is contrast to our practical experience with propofol, where the effect occurs exclusively during the time when the attention on the patient is greatest. During the endoscopic procedure, the $PcCO_2$ monitoring indicated no increased hypoventilation risk for propofol when compared with midazolam.

Insufflation of the colon with carbon dioxide (CO_2) rather than air has been shown to reduce pain and discomfort because CO_2 is rapidly absorbed by the intestinal lining. In previous studies, measurement of end tidal CO_2 ($ETCO_2$) and the mean pCO_2 demonstrated these procedures to be safe. However, no studies have used transcutaneous continuous pCO_2 monitoring, which could be valuable given the increasing use of this insufflation technique for pain relief during colonoscopies[26].

A metanalysis by Qadeer *et al*[21] showed that propofol sedation had a lower rate of cardiopulmonary complications than traditional agents used during colonoscopy procedures. This current study highlights another physiological mechanism that may be detrimental when propofol is used in larger cohorts. Therefore, assessing the $PcCO_2$ during sedation could serve as an added safety measure to detect alveolar hypoventilation.

In conclusion, hypoventilation occurs frequently during sedation for colonoscopy and is often undetected during routine pulse oximetry. A significantly higher number of patients sedated with propofol had normalized $PcCO_2$ values five minutes after sedation when compared with patients sedated with midazolam. Understanding the role of CO_2 retention will be important in increasing the further safety standards of sedation during endoscopy. More studies are required to identify and prevent hypercapnia and thus ensure the safe practice of sedation during routine gastrointestinal endoscopies.

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COMMENTS

Background

Colonoscopies are usually performed under sedation and monitored by pulse oximetry. With the increasing use of newer sedative agents such as propofol, there is an ongoing discussion about safety and monitoring requirements.

Research frontiers

The surveillance of carbon dioxide tension (e.g., measuring end tidal CO_2 by capnography) could provide more accurate information than pulse oximetry regarding ventilation impairment. Until now, little experience has been reported for transdermal CO_2 measurement systems used for this purpose.

Innovations and breakthroughs

Monitoring studies with capnography showed that hypoventilation and even short apneas that are not detected by pulse oximetry (POX) may occur during endoscopic sedation. However, an increasing body of scientific data shows that sedation during endoscopy with benzodiazepines and propofol is a safe procedure. The development of a new sensor combining transcutaneous carbon dioxide and pulse oximetry measurements provides the opportunity to explore a new monitoring method during endoscopic sedation.

Applications

The study results suggest that hypoventilation occurs often during endoscopic sedation but lasts for a shorter period if patients are sedated with propofol rather than midazolam.

Terminology

$PcCO_2$: Transcutaneous carbon dioxide tension is measured electrochemically using a Severinghaus-type sensor placed on the earlobe; POX: Pulse oximetry measures the oxygen saturation of the blood using an optical sensor.

Peer review

In this study, the authors investigated carbon dioxide accumulation after sedation with propofol or midazolam during colonoscopies. Non-invasive, continuous transcutaneous carbon dioxide tension ($PcCO_2$) monitoring was performed using a recently developed $POX/PcCO_2$ sensor that was placed at the earlobe. The results of the study show that a significantly higher number of patients sedated with propofol had normalized $PcCO_2$ values five minutes after sedation when compared with the patient group sedated with midazolam. The study is well designed and performed, and the $POX/PcCO_2$ sensor could be used in future studies rather than capnography.

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BRIEF ARTICLE

Similar fecal immunochemical test results in screening and referral colorectal cancer

Sietze T van Turenhout, Leo GM van Rossum, Frank A Oort, Robert JF Laheij, Anne F van Rijn, Jochim S Terhaar sive Droste, Paul Fockens, René WM van der Hulst, Anneke A Bouman, Jan BMJ Jansen, Gerrit A Meijer, Evelien Dekker, Chris JJ Mulder

Sietze T van Turenhout, Frank A Oort, Jochim S Terhaar sive Droste, Chris JJ Mulder, Department of Gastroenterology and Hepatology, VU University Medical Center, 1081 HV Amsterdam, The Netherlands
Leo GM van Rossum, Department of Epidemiology Biostatistics and HTA, Radboud University Nijmegen Medical Center, 6500 HB Nijmegen, The Netherlands
Robert JF Laheij, Jan BMJ Jansen, Department of Gastroenterology and Hepatology, Radboud University Nijmegen Medical Center, 6500 HB Nijmegen, The Netherlands
Anne F van Rijn, Paul Fockens, Evelien Dekker, Department of Gastroenterology and Hepatology, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands
René WM van der Hulst, Department of Gastroenterology and Hepatology, Kennemer Gasthuis, 2035 RC Haarlem, The Netherlands
Anneke A Bouman, Clinical Chemistry, VU University Medical Center, 1081 HV Amsterdam, The Netherlands
Gerrit A Meijer, Department of Pathology, VU University Medical Center, 1081 HV Amsterdam, The Netherlands
Author contributions: van Turenhout ST, van Rossum LGM, Meijer GA, Dekker E, Mulder CJJ were responsible for study concept and design; van Turenhout ST, van Rossum LGM, Oort FA, Laheij RJF, van Rijn AF, Terhaar sive Droste JS, van der Hulst RWM, Fockens P, Jansen JBMJ, Meijer GA, Dekker E, Mulder CJJ obtained and interpreted the data; Bouman AA provided technical and material support for acquisition of test analysis; van Turenhout ST, van Rossum LGM analyzed the data; van Turenhout ST, van Rossum LGM drafted the manuscript; Oort FA, Laheij RJF, van Rijn AF, Terhaar sive Droste JS, van der Hulst RWM, Fockens P, Jansen JBMJ, Meijer GA, Dekker E, Mulder CJJ critically revised the manuscript for important intellectual content; van Rossum LGM, Meijer GA, Dekker E, Mulder CJJ supervised the study; all authors approved the final version of the manuscript.
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Correspondence to: Sietze T van Turenhout, MD, Department of Gastroenterology and Hepatology, VU University Medical Center, PO Box 7057, 1081 HV Amsterdam, The Netherlands. s.vanturenhout@vumc.nl
Telephone: +31-20-4440613 Fax: +31-20-4440554
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Abstract

AIM: To improve the interpretation of fecal immunochemical test (FIT) results in colorectal cancer (CRC) cases from screening and referral cohorts.

METHODS: In this comparative observational study, two prospective cohorts of CRC cases were compared. The first cohort was obtained from 10 322 average risk subjects invited for CRC screening with FIT, of which, only subjects with a positive FIT were referred for colonoscopy. The second cohort was obtained from 3637 subjects scheduled for elective colonoscopy with a positive FIT result. The same FIT and positivity threshold (OC sensor; ⩾ 50 ng/mL) was used in both cohorts. Colonoscopy was performed in all referral subjects and in FIT positive screening subjects. All CRC cases were selected from both cohorts. Outcome measurements were mean FIT results and FIT scores per tissue tumor stage (T stage).

RESULTS: One hundred and eighteen patients with CRC were included in the present study: 28 cases obtained from the screening cohort (64% male; mean age 65 years, SD 6.5) and 90 cases obtained from the referral cohort (58% male; mean age 69 years, SD 9.8). The mean FIT results found were higher in the referral cohort (829 ± 302 ng/mL *vs* 613 ± 368 ng/mL, P = 0.02). Tissue tumor stage (T stage) distribution was dif-

ferent between both populations [screening population: 13 (46%) T1, eight (29%) T2, six (21%) T3, one (4%) T4 carcinoma; referral population: 12 (13%) T1, 22 (24%) T2, 52 (58%) T3, four (4%) T4 carcinoma], and higher T stage was significantly associated with higher FIT results ($P < 0.001$). Per tumor stage, no significant difference in mean FIT results was observed (screening *vs* referral: T1 498 ± 382 ng/mL *vs* 725 ± 374 ng/mL, $P = 0.22$; T2 787 ± 303 ng/mL *vs* 794 ± 341 ng/mL, $P = 0.79$; T3 563 ± 368 ng/mL *vs* 870 ± 258 ng/mL, $P = 0.13$; T4 not available). After correction for T stage in logistic regression analysis, no significant differences in mean FIT results were observed between both types of cohorts ($P = 0.10$).

CONCLUSION: Differences in T stage distribution largely explain differences in FIT results between screening and referral cohorts. Therefore, FIT results should be reported according to T stage.

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Key words: Screening population; Referral cohort; Fecal immunochemical test; Tumor stage distribution; Colorectal cancer

Peer reviewers: Peter Laszlo Lakatos, MD, PhD, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, H1083 Budapest, Hungary; Dr. Paul Sharp, Department of Nutrition, King's College London, Franklin Wilkins Building, 150 Stamford Street, London SE1 9NH, United Kingdom

van Turenhout ST, van Rossum LGM, Oort FA, Laheij RJF, van Rijn AF, Terhaar sive Droste JS, Fockens P, van der Hulst RWM, Bouman AA, Jansen JBMJ, Meijer GA, Dekker E, Mulder CJJ. Similar fecal immunochemical test results in screening and referral colorectal cancer. *World J Gastroenterol* 2012; 18(38): 5397-5403 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i38/5397.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i38.5397

INTRODUCTION

Colorectal cancer (CRC) is a disease well suited for population-based screening. In industrialized countries, CRC is one of the three malignancies with the highest incidence and mortality[1,2]. CRC and adenomas can cause lower gastrointestinal bleeding, and early detection by guaiac-based fecal occult blood tests (g-FOBTs) can decrease mortality[3-5]. Recently, fecal immunochemical tests (FITs) have been found to be superior over g-FOBTs[6-10].

Studies on FITs have used different designs, e.g., in terms of populations studied. Screening and referral cohorts each have their pros and cons in this respect[6,7]. Studies in screening cohorts, consisting of individuals with average-risk for CRC, best reflect the true target population. However, in most such studies, only subjects who test positive on FIT are referred for colonoscopy, which means that sensitivity and specificity cannot be determined directly[6]. In addition, the number of cases detected, particularly cancers, is usually low. Other designs include high risk or referral cohorts. Here, an important advantage is that colonoscopy is performed in all patients allowing for calculation of direct sensitivity and specificity[7]. In addition, these studies often yield more cases, allowing for more detailed subgroup analyses[7,11].

It has been suggested that conclusions from referral studies cannot be extrapolated to the screening setting[12,13]. So far, no comparative data have been published to verify or falsify this hypothesis, and arguments both in favor of, as well as against this hypothesis exist. Due to the higher pretest likelihood and presence of symptomatic individuals included in referral cohorts, the risk of work-up bias exists, limiting extrapolation to population-based screening. On the other hand, CRC stage distribution has been shown to influence sensitivity of blood-based CRC markers[14], and is likely to have a large influence on FIT results[15]. Therefore, the aim of the present study was to compare FIT results between subjects with CRC found in either a screening or a referral cohort, and determine if differences can be explained by tumor characteristics.

MATERIALS AND METHODS

Study design

The present study aimed to compare FIT results in CRC cases derived from two methodologically different cohorts. Here, CRC cases with a FIT result ⩾ 50 ng/mL from both a screening and a referral cohort were compared for mean FIT result, age, sex and tumor stage. Both these studies were initiated in the same time period in the same country, and both used the same FIT.

Setting

Screening population: The screening cohort in this study was prospectively selected from June 2006 to February 2007 by a randomized selection from a Dutch population. Details from this study are described elsewhere[6,16]. In short, eligible individuals 50-75 years of age were invited by mail to perform either a g-FOBT or a FIT ($n = 20\,623$). Participants with a positive test were offered colonoscopy in two academic centers. Exclusion criteria were institutionalization and bowel symptoms[6]. In the current study, only participants enrolled in the FIT arm ($n = 10\,322$) were included.

Referral population: The referral cohort in this study was derived from a previous and ongoing study on FIT performance, and a more detailed description can be found elsewhere[7]. In short, from June 2006 to October 2009, all ambulatory patients (aged ⩾ 18 years) scheduled for elective colonoscopy in five Dutch hospitals (including an academic center and large teaching hospital), were selected regardless of the indication for colonoscopy. Eligible subjects were invited to participate in a prospective study on FIT performance. All participants performed a FIT before bowel preparation. Until June 2008, subjects were invited to perform both a g-FOBT and an FIT[7]. In

an ongoing study after that period, subjects were invited to perform an FIT only, but twice (on two consecutive days). From the latter cohort, the FIT performed 1 d before colonoscopy was selected for the current study for comparability with the screening population. Exclusion criteria were hospitalization, colostomy, inflammatory bowel disease or total colectomy.

Outcomes and measurements

FIT: In both cohorts, an identical semi-quantitative FIT was used: OC-sensor (Eiken Chemical Co., Tokyo, Japan). No restrictions for diet or medication in the week prior to FIT were given. Participants were educated by illustrated and written instructions to sample their feces, ensuring that no contamination with water or urine occurred. In the screening population, participants were asked not to perform the test if blood was visible.

The FIT used in this study consists of a sampling tube, filled with stabilizing buffer. Participants were instructed to scrape the probe at different parts of the stool. The amount of feces that can be inserted into the sample bottle is regulated to approximately 10 mg[17]. In the referral population, subjects performed the FIT within 72 h before colonoscopy, and returned the test and informed consent form on the day of colonoscopy. All samples were placed at -5 ℃ on arrival and analyzed according to the manufacturer's instructions within 1 wk, or were frozen at -20 ℃. The analyses were performed by two experienced technicians, blinded to the clinical data[18]. In the screening population, participants were instructed to return the test by mail as soon as possible. If the test could not be returned immediately, storage in a refrigerator was advised. Again, two specially trained technicians who were blinded to the clinical data processed all samples.

All FITs were analyzed with the OC sensor MICRO desktop analyzer (Eiken Chemical Co.). The agglutination reaction is dependent on the hemoglobin concentration in the sample. A prozone effect may occur if the concentration is too high and the excess amount of antigen limits its agglutination. Measured values can then be higher or lower than the actual concentration in the sample[19]. The risk of the prozone effect gradually increases well above 1000 ng/mL. Therefore, every measurement above 1000 ng/mL was classified as 1000 ng/mL. The quantitative nature of the test was maintained, because 1000 ng/mL is at least 10 times higher than the most usual cut-off values between 50 ng/mL and 100 ng/mL.

Colonoscopy and detected malignancies: Colonoscopies were performed under conscious sedation with midazolam and fentanyl at the discretion of the endoscopist. In both cohorts, all colonoscopies were performed or supervised by experienced gastroenterologists. Colonoscopy was considered complete if the cecum was intubated with visualization of the ileocecal valve or the appendiceal orifice, or by intubation up to CRC. Incomplete colonoscopies were excluded. In addition, subjects were excluded in case of insufficient bowel cleansing, as judged by the individual endoscopist. In the screening cohort, an incomplete colonoscopy was followed by a second colonoscopy with propofol anesthesia. If necessary, a computed tomographic colonoscopy was performed followed by a second colonoscopy. If an incomplete colonoscopy in the referral cohort was followed by a complete second colonoscopy, virtual colonoscopy or x-colon within 6 mo, the results were included in analysis.

In the screening study, histology of tissue samples obtained during colonoscopy was evaluated by one experienced pathologist. In the referral cohort, lesions were evaluated according to routine procedures. In both studies, the outcome variable CRC was classified according to tissue tumor stage (T stage) of the TNM-classification (6th edition) according to the AJCC cancer staging manual[20].

Statistical analysis

The primary aim of the study was to compare mean FIT scores in CRC cases found in the referral and screening setting, with and without correction for CRC T stage. For analyses, only individuals with CRC and a FIT result $\geqslant$ 50 ng/mL were selected, because this cut-off value was used for colonoscopy referral in the screening population.

FIT scores do not follow a Gaussian curve. On average, even after correction for the prozone effect[19], the curve is considerably skewed to the left. Logarithmic transformation of the FIT scores allowed for using the *t* test, as a normal distribution was achieved. Multivariate logistic regression analysis was used to evaluate which variables could explain the differences in FIT scores between CRC patients found in the screening and referral cohorts. In logistic regression analysis, the outcome variable was mean FIT score, and the independent variables were population of origin, T stage, age, and sex. Logistic regression analysis was performed both by forward and backward selection.

Statistical analysis was performed with SAS for Windows, version 8.02 (SAS Institute Inc., Cary, NC). Two-sided P values < 0.05 were considered statistically significant.

Ethical approval and consent

In both studies, informed consent was obtained from all participants. Approval and consent from the screening arm of this study was obtained by the Dutch Health Council (2005/03WBO, The Hague, The Netherlands, www.gezondheidsraad.nl)[6]. In all centers participating in the referral arm of this study, local Medical Ethics Review Board approval was obtained prior to the start of the study[7].

RESULTS

Participants

In the screening population, 10 322 subjects were invited to FIT sampling. Of these, 6157 completed and returned the test. Five hundred and twenty-six participants were scheduled for colonoscopy because the FIT result was $\geqslant$ 50 ng/mL (i.e., positivity rate of 8.5%). In 428 patients, colonoscopy was performed, and considered complete in

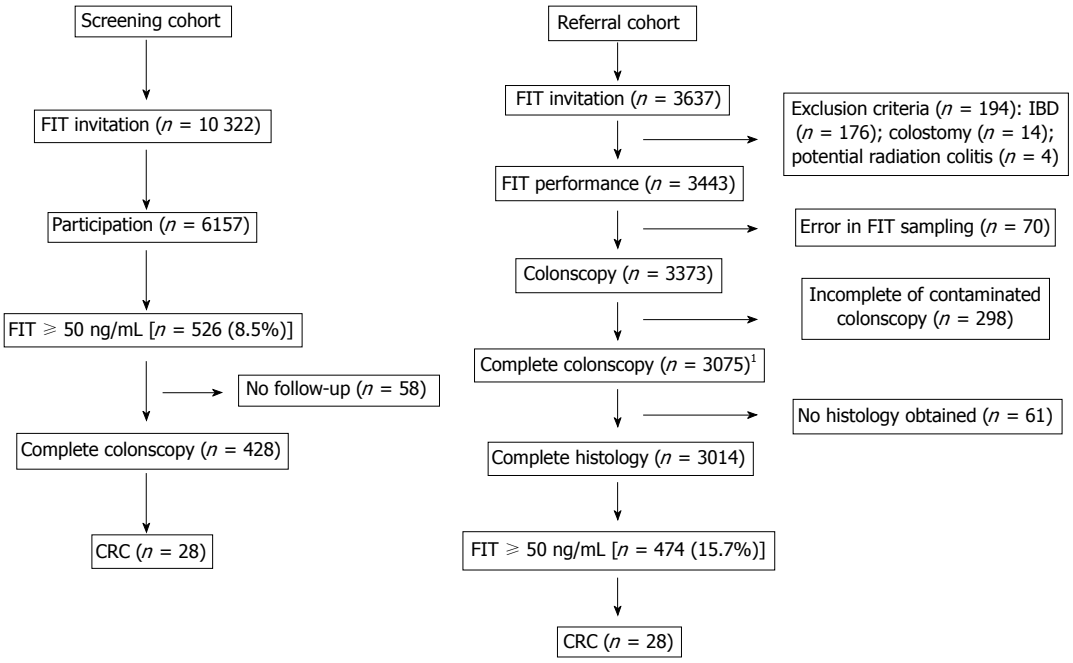


Figure 1 Study flow of the screening and referral cohort compared in the present study. [1]This includes cases in which an incomplete initial colonoscopy was followed by a second complete colonoscopy, virtual colonoscopy or x-colon. FIT: Fecal immunochemical test; CRC: Colorectal cancer; IBD: Inflammatory bowel disease.

Table 1 Primary indications for colonoscopy

Indication group	Indication for colonoscopy	*n*
Symptomatic/suspect	Weight loss	52
	Clinical suspicion of diverticulitis	23
	Clinical suspicion of IBD	40
	Abdominal pain	310
	Anemia	174
	Hematochezia	418
	Altered bowel habits	416
	Clinical or radiological suspicion of CRC	49
	Colonoscopy for polypectomy	57
	Diarrhea	115
	Constipation	71
	Total	1725
Screening/surveillance	Average risk	69
	Familial history of CRC	387
	Lynch syndrome	42
	Polyp surveillance	491
	Post CRC surveillance	157
	Total	1146
Other	Not specified/others	143
Grand total		3014

Primary indications for colonoscopy among 3014 patients included in the referral arm of this study in a comparison of fecal immunochemical test results in screening and referral colorectal cancer (CRC) cases (exclusion criteria used in the referral arm of the study were age < 18 years, hospitalization, colostomy, inflammatory bowel disease (IBD) or total colectomy).

402 (colonoscopy completion rate 94%; Figure 1). In the 26 cases in which the cecum was not visualized, a second complete colonoscopy was performed. In total, 28 cases of CRC were detected.

In the referral population, 3637 subjects were invited for participation. Six hundred and 23 subjects were excluded because of FIT sampling violating the study protocol, incomplete or contaminated colonoscopy, or missing histology of lesions found. Therefore, 3014 individuals who had a complete colonoscopy and histology remained for analysis (completion rate colonoscopy 91%, Figure 1). The indication for colonoscopy was evaluation of symptoms in 57%, screening and surveillance in 38%, and unspecified in 5% (Table 1). The positivity rate (⩾ 50 ng/mL) was 15.7%. In total, 105 subjects with CRC were found, of whom 96 (91.4%) had a positive FIT. The sensitivity of FIT for detection of CRC at cut-off values of 50 mg/mL, 75 mg/mL and 100 mg/mL was 91.4%, 90.5% and 89.5%, respectively. The respective specificity for these cut-off values was 83.7%, 85.7% and 87.0%.

In total, 124 patients with CRC and a positive FIT result were found: 28 derived from the screening population and 96 from the referral population. From the referral population, six cases were excluded because the actual T stage could not be determined due to neoadjuvant radiotherapy or palliative treatment. The mean age of the remaining 90 CRC cases from the referral cohort was significantly higher compared with the 28 cases from the screening cohort; 69 (SD 9.8) *vs* 65 (SD 6.5) years, respectively ($P = 0.04$). As expected, the proportion of males was higher in both populations and not statistically significantly different between the referred and screened population (58% and 64%, $P = 0.54$, Table 1). Other abnormalities that might cause (minor) mucosal bleeding in the colon in addition to CRC, potentially influencing the FIT results were seen in 64% of the referral and 79% (not significant) of the screening population (Table 2).

T stage distribution and FIT results

The 28 CRC cases from the screening population had a

Table 2 Colorectal cancer patient characteristics and potential explanations for positive fecal immunochemical test results in addition to colorectal cancer

	Screening population (*n* = 28)	Referral population (*n* = 90)
Male %	64	58[1]
Age (yr, mean ± SD)	65 ± 6.5	69 ± 9.8[2]
Location of CRC (% left sided)	64	62
CRC only (%)	6 (21)	32 (36)
CRC including (%)		
Advanced adenomas	16 (57)	14 (16)
Other adenomas	5 (18)	17 (19)
Other polyps	0 (0)	14 (16)
Diverticula	1 (4)	9 (10)
Hemorrhoids	0 (0)	4 (4)

[1]χ^2 test: $P = 0.54$; [2]t test: $P = 0.04$. The percentages of other abnormalities in addition to colorectal cancer (CRC) in the referral population totaled 65% due to rounding. The total percentage of CRC cases with additional pathology was 64%.

mean FIT score of 613 ng/mL (SD 368 ng/mL), which was significantly lower ($P = 0.02$) than the mean FIT score of the 90 CRC cases from the referral population 829 ng/mL (SD 302 ng/mL, Table 3).

The CRC stage distribution was different between the populations: early stages were more frequently found in the screening population (Figure 2). Of the 28 cases from the screening population, 13 cases were classified as T1 (46%), eight as T2 (29%), six as T3 (21%) and one as T4 carcinoma (4%). In the referral population, 12 patients had stage T1 tumor (13%), 22 stage T2 (24%), 52 stage T3 (58%) and four stage T4 (4%).

After stratifying mean FIT scores by T stage, none of the tumor stages had a significant difference in FIT results (Table 2): T1, $P = 0.22$; T2, $P = 0.79$; T3, $P = 0.13$. There was only one T4 case in the screening population, therefore, T4 cases were combined with T3 cases. Again, for the combined T3 and T4 stage category, no significant difference in FIT score was seen between both populations ($P = 0.19$).

Logistic regression analysis

By univariate analysis, FIT results from the referral cohort were significantly higher compared with the screening cohort ($P < 0.01$). However, after adding T stage to the model, the difference in FIT results lost statistical significance ($P = 0.10$). The P values per T stage were 0.23 (T1), 0.79 (T2), and 0.11 (T3/T4). By multivariate analysis including the variables sex, age, T, N and M stage, only T stage ($P < 0.001$) and (marginally) age ($P = 0.05$) could significantly explain the differences between the screening and referral cohorts.

DISCUSSION

The current study compared FIT results in individuals with CRC from two different prospective study designs, i.e., a population-based screening study and a referral cohort, to study the uncertainty about the link between the results obtained from these kind of studies. Cases of CRC from the screening cohort were found to have significantly lower FIT results compared to those from the referral cohort, but after stratifying for tissue tumor stage, no difference remained. In the screening cohort, 75% (21/28) and in the referral cohort only 38% of cases had a T1 or T2 tumor (34/90). Logistic regression analysis confirmed that not the type of population, but only T stage and to a lesser extent age could explain the differences in FIT results of CRC patients between these screening and referral cohorts.

The results of this study are relevant for the evaluation of CRC screening tests and guidance of future study designs. Several studies in screening populations have been performed comparing, e.g., FIT with g-FOBT[6,9,12,21]. However, despite the large number of screened individuals in these studies, the absolute number of individuals with CRC was relatively low, hampering subgroup analysis. Furthermore, colonoscopy was missing in subjects with a negative FIT, impeding calculation of sensitivity and specificity. Indeed, for the investigation of the performance of a screening test like FIT, the ultimate prospective study design would contain full colonoscopic evaluation of all participants. However, in a screening population, this is considered unethical or unfeasible because the capacity and/or funds are lacking. In referral populations, FIT negatives do indeed all undergo colonoscopy, and in addition, in much less time and at a substantially lower cost, a much larger number of CRC patients can be included. This enables, e.g., more elaborate subgroup analysis of early stage CRC. The present study shows that tumor stage is the major contributor to the outcomes of FIT between cohorts. Possible differences in characteristics between the cohorts did not have much influence on FIT outcomes. It is indicated here that, if correction or stratification for CRC tumor stage distribution is applied, both screening as well as referral cohorts can be used to answer several important screening-related research questions. Research questions on accuracy of screening tests with sufficient power, could therefore initially be explored using referral populations. In line with Tao and colleagues, who found that sensitivity of blood-based CRC markers is dependent on tumor stage[14], results for test characteristics should be presented per tumor stage. By merging results from different sources, the strength of the evidence available will be enlarged.

Some considerations need to be discussed for proper interpretation of the present results. For evaluation of sensitivity and specificity, data from referral studies could be extrapolated, or the incidence of interval cancers could be used as false negatives. The latter requires intensive follow-up over many years before sensitivity can be estimated; time in which tumors may further evolve. Second, the number of screening cases is limited, although comparable with other screening studies[9,12,21]. This limits the power to determine any existing differences. However, from Figure 2 and Table 2 it is clear that it is unlikely that adding more screening cases could change the results substantially. Supported by the results from other

Table 3 Fecal immunochemical test results in patients with colorectal cancer derived from a screening and referral cohort according to tissue tumor stage

	Population	*n*	mean ± SD	25th %	Median	75th %	*P* value[1]
All colorectal cancer cases	Screening	28	613 ± 368	283	662	1000	0.02
	Referral	90	829 ± 302	709	1000	1000	
T1	Screening	13	498 ± 382	79	384	871	0.22
	Referral	12	725 ± 374	428	1000	1000	
T2	Screening	8	787 ± 303	559	936	1000	0.79
	Referral	22	794 ± 341	550	1000	1000	
T3	Screening	6	563 ± 368	269	454	1000	0.13
	Referral	52	870 ± 258	888	1000	1000	
T4	Screening	1	NA	NA	NA	NA	NA
	Referral	4	793 ± 415	586	1000	1000	

[1]*t* test after logarithmic transformation because of non-normality of fecal immunochemical test results (ng/mL). All results ⩾ 1000 ng/mL were classified as 1000 ng/mL. T: Tumor stage; NA: Not available. (i.e., values like mean and median having a single observation are meaningless and a *t* test cannot be performed because no error term can be estimated for a single observation).

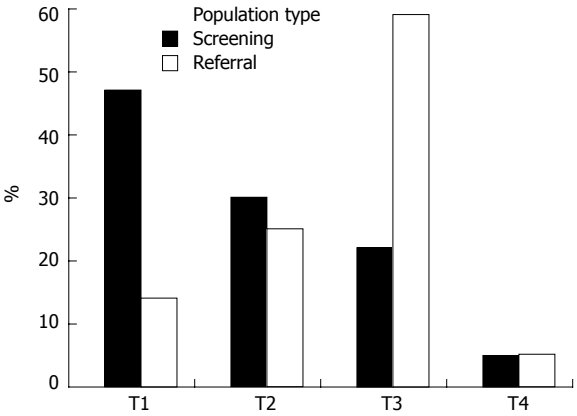


Figure 2 Colorectal cancer tumor stage distribution in screening (*n* = 28) and referral (*n* = 90) populations in studies on diagnostic performance of an fecal immunochemical test. Difference between screening and referral $P < 0.001$.

studies[13,16,17,22], CRC was considered the major contributor to the overall FIT results. Still, it cannot be excluded that other sources of bleeding (like minor polyps) could have contributed to the overall FIT result. In addition, although probably limited considering the moderate temperatures in the Netherlands, time lag between sampling and analysis could have influenced FIT results found[23]. In this study, the existing difference between both cohorts could therefore be even less, because time to analysis in the screening cohort was on average somewhat longer. Finally, in the screening cohort, no information about preoperative radiotherapy was available and therefore tumor stage could have been underestimated in a few cases. In summary, the correction for other potential bleeding sources, time lag to analysis, and radiotherapy would even decrease the differences between the referral and screening cohorts and therefore support our conclusions.

The present study aimed to compare test performance in two study designs, each with pros and cons, knowing the essential differences between the two cohorts. It can be concluded that T stage reflects the majority of the differences in overall FIT results between the two studied cohorts.

In conclusion, apparent differences in FIT results between screened and referred CRC patients can be attributed to tissue tumor stage. Based on these findings, we conclude that results from both cohorts could strengthen the evidence available. Using referral populations for studying FIT, and potentially also new CRC screening tests, can be useful to stimulate progress in CRC research. Here, sensitivity and specificity should be studied as these measures are independent of the prevalence of the disease[24], and test characteristics should be stratified by tumor stage. This will be of particular benefit in research questions that require large numbers of cases or colonoscopy confirmation in all individuals, and do not seek predictive values as outcome.

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COMMENTS

Background

Colorectal cancer (CRC) is a disease well suited for population-based screening. Subjects who test positive on fecal immunochemical tests (FITs) should be referred for colonoscopy. FIT performance has been studied in two different types of populations. In studies in screening cohorts, only subjects who test positive on FIT are referred for colonoscopy. Sensitivity and specificity cannot be calculated directly. In addition, the number of cases detected is usually low. Designs with referral cohorts do not study average but high-risk individuals. However, as colonoscopy is performed in all subjects direct sensitivity and specificity can be calculated. In addition, in referral populations, more cases are found. The aim of the present study was to compare FIT results between subjects with CRC found in a screening and a referral cohort.

Research frontiers

FITs detect occult human blood, what might be derived from adenomas or CRC. FITs are more sensitive than guaiac fecal occult blood tests, by which screening has been shown to decrease CRC-related mortality. However, exact FIT characteristics are the subject of debate.

Innovations and breakthroughs

The present study is the first to compare results obtained from both screening and referral populations to gain insight into the comparability of results derived from both study designs. It was shown that referral populations have a different tumor stage (T stage) distribution compared to screening populations (i.e., a higher percentage of high T stage cancers). This was accompanied by higher mean FIT results. After correction for T stage, mean FIT results were similar in both populations.

Applications

Apparent differences in FIT results between screened and referred CRC patients can be attributed to tissue tumor stage. Results from both cohorts could strengthen the evidence available. Using referral populations for studying FIT, and potentially also new CRC screening tests, can be useful to stimulate progress in CRC research, when test characteristics are stratified by tumor stage.

Peer review

This is an important study comparing the appropriate interpretation of FIT in screening population and in patients referred for colonoscopy regardless of the indication. According to the authors' conclusion differences in T-stage distribution largely explained differences in FIT results between screening and referral cohorts. Therefore the absolute value of the FIT results should be reported according to T-stage.

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BRIEF ARTICLE

Different risk factors influence peptic ulcer disease development in a Brazilian population

Rodrigo Buzinaro Suzuki, Rodrigo Faria Cola, Larissa Tranquilino Bardela Cola, Camila Garcia Ferrari, Fred Ellinger, Altino Luiz Therezo, Luis Carlos da Silva, André Eterovic, Márcia Aparecida Sperança

Rodrigo Buzinaro Suzuki, Rodrigo Faria Cola, Larissa Tranquilino Bardela Cola, Department of Genotyping, Hemocenter, Marilia Medical School, Marilia 17519-030, São Paulo, Brazil
Rodrigo Buzinaro Suzuki, André Eterovic, Márcia Aparecida Sperança, Center for Natural and Human Sciences, Universidade Federal do ABC, Santo André 09210-170, São Paulo, Brazil
Camila Garcia Ferrari, Department of Molecular Biology, Marilia Medical School, Marilia 17519-030, São Paulo, Brazil
Fred Ellinger, Altino Luiz Therezo, Luis Carlos da Silva, Department of Pathology, Marilia Medical School, Marilia 17519-030, São Paulo, Brazil
Author contributions: Suzuki RB contributed to the collection and processing of biopsy samples, epidemiological and molecular comparative analyses; Cola RF and Cola LTB contributed with analytic tools and molecular analysis; Ferrari CG contributed with molecular analysis; Ellinger F, Therezo AL and Silva LC contributed equally to the histopathological analysis; Eterovic A contributed with statistical analysis; and Sperança MA designed the research, wrote the paper and contributed to all comparative analyses.
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Correspondence to: Márcia Aparecida Sperança, PhD, Center for Natural and Human Sciences, Universidade Federal do ABC, Rua Santa Adélia, 166 Bloco A, Torre 3, 6º andar Sala 625, Bairro Bangu 09210-170, Santo André, São Paulo, Brazil. marcia.speranca@ufabc.edu.br
Telephone: +55-11-49968373 Fax: +55-11-49960090
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Abstract

AIM: To investigate age, sex, histopathology and *Helicobacter pylori* (*H. pylori*) status, as risk factors for gastroduodenal disease outcome in Brazilian dyspeptic patients.

METHODS: From all 1466 consecutive dyspeptic patients submitted to upper gastroscopy at Hospital das Clinicas of Marilia, antral biopsy specimens were obtained and subjected to histopathology and *H. pylori* diagnosis. All patients presenting chronic gastritis (CG) and peptic ulcer (PU) disease localized in the stomach, gastric ulcer (GU) and/or duodenal ulcer (DU) were included in the study. Gastric biopsies (n = 668) positive for *H. pylori* by rapid urease test were investigated for vacuolating cytotoxin A (*vacA*) medium (m) region mosaicism by polymerase chain reaction. Logistic regression analysis was performed to verify the association of age, sex, histopathologic alterations, *H. pylori* diagnosis and *vacA* m region mosaicism with the incidence of DU, GU and CG in patients.

RESULTS: Of 1466 patients submitted to endoscopy, 1060 (72.3%) presented CG [male/female = 506/554; mean age (year) ± SD = 51.2 ± 17.81], 88 (6.0%) presented DU [male/female = 54/34; mean age (year) ± SD = 51.4 ± 17.14], and 75 (5.1%) presented GU [male/female = 54/21; mean age (year) ± SD = 51.3 ± 17.12] and were included in the comparative analysis. Sex and age showed no detectable effect on CG incidence (overall χ^2 = 2.1, P = 0.3423). Sex [Odds ratios (OR) = 1.8631, P = 0.0058] but not age (OR = 0.9929, P = 0.2699) was associated with DU and both parameters had a highly significant effect on GU (overall χ^2 = 30.5, P < 0.0001). The histopathological results showed a significant contribution of ageing for both atrophy (OR = 1.0297, P < 0.0001) and intestinal metaplasia (OR = 1.0520, P < 0.0001). Presence of *H. pylori* was significantly associated with decreasing age (OR = 0.9827, P < 0.0001) and with the incidence of DU (OR = 3.6077, P < 0.0001). The prevalence of m1 in DU was statistically significant (OR = 2.3563, P = 0.0018) but not in CG (OR = 2.678, P = 0.0863) and GU (OR = 1.520, P= 0.2863).

CONCLUSION: In our population, male gender was a risk factor for PU; ageing for GU, atrophy and metapla-

sia; and *H. pylori* of *vacA* m1 genotype for DU.

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Key words: *Helicobacter pylori*; Gastric ulcer disease; Duodenal ulcer disease; Gastric atrophy; *Helicobacter pylori* vacuolating cytotoxin A medium region mosaicism

Peer reviewer: Mitsuo Shimada, Professor, Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a Gram-negative microaerophylic bacterium, is associated with a broad spectrum of digestive tract diseases such as chronic gastritis, gastric and duodenal ulcers, gastric cancer and lymphoproliferative disorders[1]. *H. pylori* infection prevalence and clinical outcome of the colonized patients varies according to several considerations including bacterial factors and host and environmental characteristics such as age, ethnic group, genera, geography and socioeconomic conditions[2].

The role of *H. pylori* in the interaction with the host has an impact on the pattern and severity of gastritis and its clinical outcome[3]. The physiologic mechanisms involved in these *H. pylori*-induced pathological differences are still unknown; however, one of the major bacterium virulence factors, the vacuolating cytotoxin A (*vacA*), seems to be involved. The vacA protein encoded by the polymorphic *H. pylori vacA* gene, is produced and secreted by all bacterium strains and induces the formation of intracellular vacuoles in epithelial cell lines *in vitro*[4-6]. Polymorphism of the *vacA* gene is distributed in three principal regions: the signal (s), intermediate (i), and middle (m) regions, each being divided in two main types, numbered 1 and 2[7,8], which are differently associated with several mechanisms of pathogenicity *in vitro* and *in vivo*[6,9-15]. The s1, i1, and m1 types have been shown to be independently associated with more severe forms of *H. pylori*-induced diseases[8,16].

Studies conducted in several countries have shown that type 1 and 2 alleles of *vacA* polymorphisms are both widespread in all populations examined, except in the Japanese, among whom type 2 alleles are rare[17,18]. Thus, outside Asia, *vacA*-type 1 and *cagA*-positive *H. pylori* strains are more frequently associated with severe *H. pylori*-induced peptic ulcer diseases than *vacA*-type 2 *cagA*-negative bacterium strains[7,19-22]. In Brazil, a country of continental dimensions, this association has been observed in children[23,24] but is controversial in the adult population[25-27].

Environmental and demographic data also interfere with the pathophysiology of *H. pylori*-associated gastric diseases. In the adult population of Brazil, *H. pylori* infection can range from 60%-90%[28-31]. In Marilia, a city of São Paulo State, the serological prevalence of *H. pylori* determined in blood donors was 57% and a risk factor associated with IgG and/or IgA *H. pylori* antibodies was low educational level[32]. Considering the large geographical dimensions of Brazil, with its regionally specific socioeconomic and cultural conditions reflected by the high and variable prevalence of *H. pylori*, there are few epidemiological studies on gastric diseases.

So far, all comparative Brazilian studies on gastric disease epidemiology, *H. pylori* prevalence and *vacA* gene mosaicism have generally been carried out on small populations and on *H. pylori* strains isolated in culture. Therefore, considering that gastric and duodenal ulcer diseases depend on different physiological trigger mechanisms, we comparatively investigated the role of age, gender, histopathologic antral gastric alterations and the *H. pylori* status, including the *vacA* m region mosaicism detected directly in biopsies, as risk factors for gastric ulcer (GU), duodenal ulcer (DU) and chronic gastritis (CG) in patients consecutively attending at Hospital das Clinicas of Marilia, São Paulo, Brazil, during a period of four years.

MATERIALS AND METHODS

Patients

Adult patients (n = 1466) resident in Marilia city, São Paulo State, Brazil, aged 19 to 91 years, underwent esophagogastroduodenoscopy (EGD) for upper abdominal pain or dyspeptic symptoms from January 2003 through December 2006 at the Gastroenterology Outpatient Clinic of the Hospital das Clínicas of Marília Medical School. All who presented GU, DU (by endoscopy) and/or CG (by histology), were enrolled in this study.

Endoscopy and biopsies

The EGD was accomplished by fibroendoscope (GIF-XP20, GIF-XQ20) or video-endoscope (GIF-100), both from Olympus Medical Systems, Shinjuku-ku, Tokyo, Japan. Gastric or duodenal ulcer diagnosis was defined by endoscopy and two fragments of the antrum were collected to perform rapid urease (RUT) and histopathological tests. The biopsy used for RUT was further submitted to DNA extraction. The protocol used is in agreement with the Helsinki Declaration and was approved by the Ethical Committee in Human Research from Marilia Medical School, under reference number 388/01.

Histology

One antral specimen was fixed in 40 g/L of formaldehyde and embedded in paraffin. Sections were Giemsa stained for *H. pylori* evaluation and were stained with he-

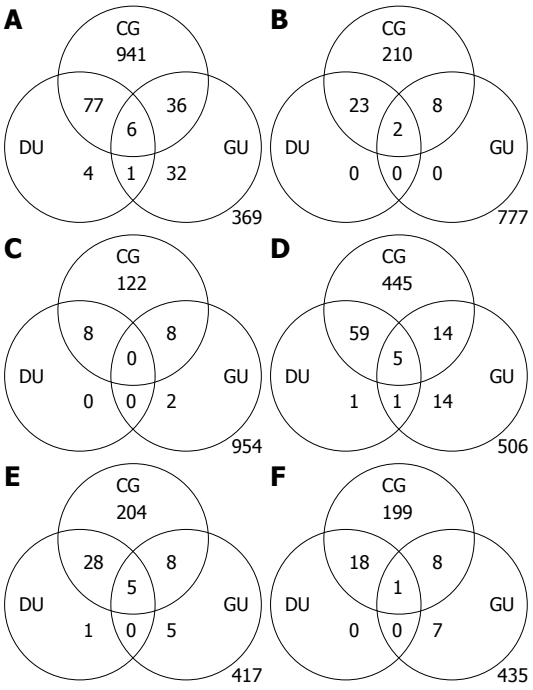


Figure 1 Incidence of chronic gastritis, duodenal ulcer and gastric ulcer disease and distribution of the investigated independent variables. A: Incidence of gastric diseases in patients who underwent endoscopy (n = 1466); B: Distribution of the histopathological parameters of atrophy (n = 1020); C: Intestinal metaplasia (n = 1094); D: Positive histological diagnosis of *Helicobacter pylori* (*H. pylori*) (n = 1045); E: *H. pylori vac*A m1 genotypes (n = 668); F: *H. pylori vacA* m2 genotypes (n = 668). CG: Chronic gastritis; DU: Duodenal ulcer; GU: Gastric ulcer; *vacA*: Vacuolating cytotoxin A. Values outside circles are negative tests.

matoxylin and eosin for assessment of histopathologic alterations according to the Sydney classification[33].

Helicobacter pylori vacA genotyping

The same biopsy used for RUT was submitted to DNA extraction with the employment of the GFx DNA extraction kit purchased from Amersham/Pharmacia Biotec, following the manufacturer's instructions. DNA was quantified in agarose gel electrophoresis using the Invitrogen low mass ladder and 50-100 μg of total DNA were used in the polymerase chain reaction (PCR) reactions with the oligonucleotides[34]: MA sense (5'CACAGCCACTTTYAATAACGA3') and MB antisense (5' CGTCAAAATAATTCCAAGGG3'), which amplify a fragment of 400 bp or 476 bp corresponding to the *vacA* m regions 1 and 2, respectively. PCR condition was 94 ℃ for 5 min followed by 40 cycles of 94 ℃ 1 min/60 ℃ 1 min/72 ℃ 1 min and one cycle at 72 ℃ 7 min, with a total volume of 25 μL containing 1 × PCR buffer, 200 μmol dNTPs, 2.0 mmol $MgCl_2$, 1 μmol of each oligonucleotide, 1.25 U Taq DNA Polimerase Platinum Brazil (Invitrogen). In all PCR reactions a negative and a positive control were used corresponding to, respectively, sterile water and *H. pylori vacA* m1 and/or m2 PCR positive gastric biopsies. The products of PCR reactions were resolved in 15 g/L agarose gels, stained with ethidium bromide and photographed under ultraviolet light.

Statistical analysis

Incidences of CG, DU, and GU in patients submitted to endoscopy were investigated using age (in years) and sex as independent factors. Only for patients who developed at least one of these diseases, the role of age, sex, CG, GU, and DU were verified as independent factors to evaluate the presence of atrophy, intestinal metaplasia, *H. pylori* and *vacA* m1 and m2 genotypes, each assessed individually. Incidences were coded as "1" and absences (of evidence) as "0" (males were also coded as "1", females as "0"). Cases without the complete records needed for each analysis were discarded. Multivariable screenings were performed by additive logistic regression models for detection of significant effects of the independent factors on each response variable. New models were made after discarding irrelevant variables and significant parameters of these last models were used to describe the relationship among factors and responses by means of logit functions. Critical P-values were considered after a Bonferroni correction based on the number of similar tests. All logistic regression analyses were performed using a free on-line device for Logistic Regression calculation provided by Pezzulo (2012)[35].

RESULTS

Among 1466 patients submitted to endoscopy, 1060 (72.3%) presented CG [male/female = 506/554; mean age (year) ± SD = 51.2 ± 17.81], 88 (6.0%) presented DU [male/female = 54/34; mean age (year) ± SD = 51.4 ± 17.14], and 75 (5.1%) presented GU [male/female = 54/21; mean age (year) ± SD = 51.3 ± 17.12], and were included in the comparative analysis. More than one of these diseases was presented by 120 (8.2%) individuals and 369 (25.2%) patients were free of them (Figure 1). Most of the other endoscopic and histopathologic alterations were related to gastroesophageal tract diseases (data not shown). Mean age and gender of the included CG, GU and GC patients are summarized.

Distribution of atrophy, intestinal metaplasia, *H. pylori* histological diagnosis and *vacA* m region mosaicism in patients presenting CG, DU and GU are summarized in Figure 1.

Atrophy and intestinal metaplasia were investigated among antral gastric biopsies of 1020 and 1094 patients, respectively, with 243 (23.8%) positive for atrophy and 140 (12.8%) for intestinal metaplasia.

Detection of *H. pylori* was performed directly from biopsy specimens by two different tests: histology and RUT. Histology is the gold standard *H. pylori* diagnostic test employed in our clinical routine which together with histopathological analysis is used to decide for *H. pylori* eradication therapy. RUT showed a very low positive predictive value for *H. pylori*-associated gastric diseases and a high discrepancy when compared to histology; consequently these data were excluded from the study (data not shown). Among 1045 patients investigated for *H. pylori* by histology, 539 (51.6%) were positive.

Among 668 biopsies of patients positive for RUT in-

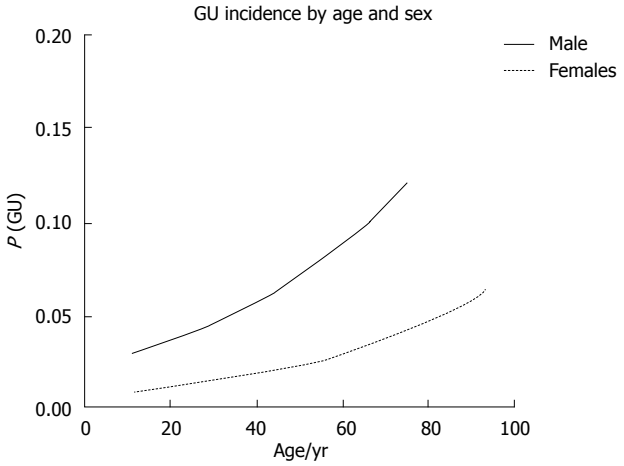


Figure 2 Logistic regression curves of age and sex as independent factors to explain gastric ulcer disease incidence. The model has a significant fit, with contributions of both coefficients (χ^2 = 30.5, P < 0.0001). Additional test for the age/sex interaction had a nonsignificant result (β_3 = -0.011, SE = 0.0161, P = 0.4844, OR = 0.989, CI- = 0.958, CI+ = 1.021). OR: Odds ratios; CI: Confidence intervals; GU: Gastric ulcer.

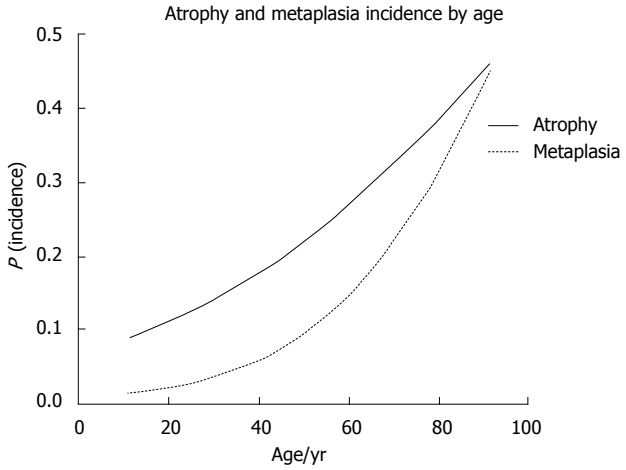


Figure 3 Logistic regression curves of age as an independent factor to explain atrophy and intestinal metaplasia incidences. All patients who underwent endoscopy and who had at least one of the three diseases were included in the analysis. Both models had highly significant fits (atrophy OR = 1.0297, P < 0.0001; intestinal metaplasia OR = 3.6077, P < 0.0001). OR: Odds ratios.

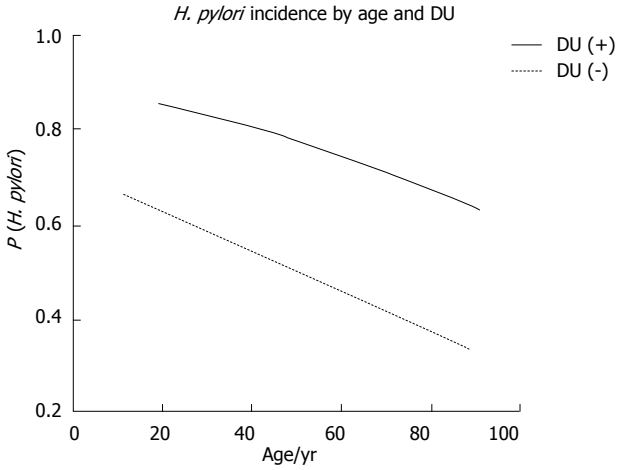


Figure 4 Logistic regression curves of age and duodenal ulcer disease as independent factors to explain *Helicobacter pylori* incidence. The model had a highly significant fit (age OR = 0.9827, P < 0.0001; DU OR = 3.6077, P < 0.0001). A posterior test for the age/DU interaction had a nonsignificant result (β_3 = 0.0085, SE = 0.0168, P = 0.6142, OR = 1.0085, CI- = 0.9758, CI+ = 1.0423). DU: Duodenal ulcer; OR: Odds ratios; CI: Confidence intervals; *H. pylori*: *Helicobacter pylori*.

vestigated for *vacA* m region mosaicism by PCR directly on biopsy specimens, 484 were positive, with 251 (51.8%) of m1 and 233 (48.2%) of m2 genotypes.

Results of the additive logistic regression models to evaluate the contribution of age and sex to the incidence of CG, DU and GU among patients who underwent gastroscopy are summarized in Table 1. Sex and age showed no detectable effect on CG incidence (overall χ^2 = 2.1, P = 0.3423). Sex (OR = 1.8631, P = 0.0058) but not age (OR = 0.9929, P = 0.2699) affected DU, and both parameters had a highly significant association with GU (overall χ^2 = 30.5, P < 0.0001), with contributions of both coefficients (age OR = 1.0233, P = 0.0017; sex OR = 3.0790, P < 0.0001). A posterior test for the age/sex interaction showed it to be nonsignificant (OR = 0.9888, P = 0.4844). The incidence of GU increased with age and further, at a given age, males had a higher probability of developing GU (Figure 2).

Additive logistic regression models were constructed to investigate the contribution of age, sex, CG, DU and GU to atrophy, intestinal metaplasia, *H. pylori* presence and *vacA* m1 and m2 mosaicism (Table 2).

The results for histopathological parameters showed a significant contribution of age for both atrophy (OR = 1.0297, P < 0.0001) and intestinal metaplasia (OR = 1.0520, P < 0.0001). Atrophy incidence increased with age (Figure 3) and a new model with only age as the independent factor also had a highly significant fit (overall χ^2 = 35.2, P < 0.0001; age OR = 1.0273, P < 0.0001) though more parsimonious (Table 2). In the case of intestinal metaplasia, it was necessary to check for the contribution of CG, which was discarded after the Bonferroni correction. A new model with only age as the independent factor also had a highly significant fit (overall χ^2 = 71.7, P < 0.0001; age OR = 1.0509, P < 0.0001) though more parsimonious (Table 2). Intestinal metaplasia increased with age, but at a lower level than atrophy for a given age (Figure 3).

Presence of *H. pylori* was significantly associated with age (OR = 0.9827, P < 0.0001) and occurrence of DU (OR = 3.6077, P < 0.0001) (Table 3). A new model with only age and DU as independent factors also had a highly significant fit (overall χ^2 = 47.8, P < 0.0001; age OR = 0.9826, P < 0 .0001; DU OR = 3.5063, P < 0.0001) but was more parsimonious (Table 3). An additional model showed that age/DU had no effect on the presence of *H. pylori* (β_3 = 0.0085, SE = 0.0168, P = 0.6142, OR = 1.0085, CI- = 0.9758, CI+ = 1.0423). *H. pylori* incidence decreased with age and DU incidence contributed to higher probabilities in developing detectable levels of the bacterium, despite individual age (Figure 4).

The five independent factors studied for the *H. pylori* *vacA* m1 and m2 genotypes resulted in nonsignificant ad-

Table 1 Additive logistic regression models to evaluate the contribution of age and sex among patients who underwent gastroscopy (n_{total} = 1466)

Factor		CG	DU	GU
Overall	n (+)	1060	88	75
	χ^2 (v = 2)	2.14	8.99	30.51
	P	0.3423	0.0112[1]	< 0.0001[1]
	β_0	0.738	-2.724	-4.836
Age	β_1	0.003	-0.007	0.023
	SE	0.0034	0.0065	0.0073
	P	0.3511	0.2699	0.0017[1]
	OR	1.003	0.993	1.023
	CI-	0.997	0.980	1.009
	CI+	1.010	1.006	1.038
Sex	β_2	0.124	0.622	1.125
	SE	0.1102	0.2256	0.2636
	P	0.2598	0.0058[1]	< 0.0001[1]
	OR	1.132	1.863	3.079
	CI-	0.912	1.197	1.837
	CI+	1.405	2.899	5.162

[1]Significant effects. n_{total}: Number of investigated cases; n (+): Number of positive cases; CG: Chronic gastritis; DU: Duodenal ulcer; GU: Gastric ulcer; OR: Odds ratios; CI: Confidence intervals. Critical P-values were adopted after a Bonferroni correction for the number of similar tests: P = 0.05/3 = 0.0167 for overall models; and P = 0.05/2 = 0.025 for each independent factor.

Table 2 Logistic regression models to evaluate the contribution of age, sex, chronic gastritis, duodenal ulcer

Factor		Atrophy	Metaplasia	*H. pylori*	m1	m2
Overall	n_{total}	1020	1094	1045	668	668
	n (+)	243	140	539	251	233
	χ^2	35.18	71.67	47.83	9.88	5.85
	v	1	1	2	1.00	1
	P	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	0.0017[1]	0.0156
	β_0	-2.614	-4.733	0.881	-0.589	-0.038
Age	β_1	0.027	0.050	-0.018	-	-0.012
	SE	0.0047	0.0063	0.0039	-	0.0048
	P	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	-	0.0162
	OR	1.027	1.051	0.983	-	0.989
	CI-	1.018	1.038	0.975	-	0.979
	CI+	1.037	1.064	0.990	-	0.998
DU	β_2	-	-	1.255	0.857	-
	SE	-	-	0.2702	0.2739	-
	P	-	-	< 0.0001[1]	0.0018[1]	-
	OR	-	-	3.506	2.356	-
	CI-	-	-	2.065	1.377	-
	CI+	-	-	5.954	4.031	-

[1]Significant effects after Bonferroni correction. n_{total}: Number of investigated cases; n (+): Number of positive cases; CG: Chronic gastritis; DU: Duodenal ulcer; OR: Odds ratios; CI: Confidence intervals; *H. pylori*: *Helicobacter pylori*.

ditive regression logistic models after Bonferroni correction. However, the evaluation of the contribution of the variables indicates a possible effect of DU (Table 3). A new model with only DU as the independent factor had a highly significant fit (overall χ^2 = 9.9, P = 0.0017) (Table 2). The incidence probability of m1 in DU patients was 0.5667 and in DU negative patients was 0.3569 (OR = 2.3563, P = 0.0018). In the case of m2, the evaluation of the variable contributions indicated a possible effect of age (Table 2). A new model with only age as the independent factor also had a nonsignificant fit, and was discarded by the adopted critical P-value (overall χ^2 = 5.8, P = 0.0156; age OR = 0.9885, P = 0.0162) (Table 2).

DISCUSSION

In order to verify the differential contribution of age, gender, histopathological outcome, *H. pylori* and *vacA* m region mosaicism with incidence of PU and CG, we investigated comparatively all cases of DU (88), GU (75) and CG (1060) found after analysis of 1466 patients consecutively submitted to gastroscopy in Hospital das Clinicas of Marilia, São Paulo, Brazil, over four years.

The most prevalent gastric disease found in our consecutive dyspeptic patients was CG (72.3%) followed by gastroesophageal alterations not included in this study. Peptic ulcer disease was prevalent in 11.1% of the patients, with 6.0% of GU and 5.1% of DU. Male gender had a statistically significant association with both PU incidences. Our results are in accordance with research done in the southern region of Brazil where DU and GU had a similar prevalence and were associated with male gender[36]. However, in a recent large scale PU epidemiological study carried out in a tertiary care hospital in another city of São Paulo State, Brazil, the prevalence of DU was four times higher than GU[37] and in this population there was a significant predominance of woman in the PU group[38]. These regional differences reinforce the specificity of risk factors associated with severe gastric diseases and the need to perform local investigations to improve health care strategies.

There are differences in the gastrointestinal physiological modifications that lead to gastric and duodenal ulcers, whose causes are multifactorial and also related to population characteristics and to specific gastroduodenal alterations due to association of *H. pylori* with host mucosa[39]. In our study, increasing age was a risk factor for the development of GU. The mean age of GU and DU patients in the Brazilian ulcer study performed in a hospital in São Paulo also differed significantly, being higher in GU[37]. Thus, more epidemiological studies have to be done in order to identify the risk factors associated with age and the onset of GU in the Marilia population.

The eradication of *H. pylori* infection cured both gastric and duodenal ulcers, and the cure rates are similar, suggesting that *H. pylori* is the key factor in peptic ulcer diseases independent of the ulcer site[40]. However, a number of studies have shown the participation of other risk factors such as smoking, alcohol intake, and nonsteroidal antiinflammatory drug (NSAID) use in the etiology of PU, which are principally associated with GU[39,41,42]. In our study the prevalence of *H. pylori* infection observed by histology was significantly higher in DU than in GU and CG (P < 0.0001). Also, in the Danish epidemiologic PU study, IgG against *H. pylori* was higher in DU (87.2%) when compared to GU (60%) patients. In another city of São Paulo, there was also

Table 3 Additive logistic regression models to evaluate the contribution of age, sex, chronic gastritis, duodenal ulcer and gastric ulcer disease

Factor		Atrophy	Metaplasia	*H. pylori*	m1	m2
Overall	n_{total}	1020	1094	1045	668	668
	n(+)	243	140	539	251	233
	$\chi^2(v = 5)$	64.34	78.63	50.14	13.46	10.64
	P	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	0.0194[1]	0.0591[1]
	β_0	-24.434	-6.616	0.832	-1.448	-0.225
Age	β_1	0.029	0.051	-0.017	-0.003	-0.012
	SE	0.0047	0.0064	0.0039	0.0048	0.0048
	P	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	0.5646	0.0139[1]
	OR	1.030	1.052	0.983	0.997	0.988
	CI-	1.020	1.039	0.975	0.988	0.979
	CI+	1.039	1.065	0.990	1.007	0.998
Sex	β_2	0.064	0.101	-0.179	0.035	-0.327
	SE	0.1530	0.1906	0.1283	0.1635	0.1659
	P	0.6775	0.5969	0.1629	0.8320	0.0485
	OR	1.066	1.106	0.836	1.035	0.721
	CI-	0.790	0.761	0.650	0.752	0.521
	CI+	1.438	1.607	1.075	1.426	0.998
CG	β_3	21.676	1.807	0.135	0.985	0.378
	SE	> 100	0.8427	0.4533	0.5742	0.5710
	P	0.9978	0.0320	0.7661	0.0863	0.5082
	OR	> 100	6.090	1.144	2.678	1.459
	CI-	0.000	1.168	0.471	0.869	0.477
	CI+	> 100	31.761	2.782	8.253	4.469
DU	β_4	0.552	-0.313	1.283	0.856	-0.175
	SE	0.2658	0.3972	0.2712	0.2771	0.2931
	P	0.0377	0.4308	< 0.0001[1]	0.0020[1]	0.5496
	OR	1.737	0.731	3.608	2.353	0.839
	CI-	1.032	0.336	2.120	1.367	0.472
	CI+	2.924	1.593	6.139	4.050	1.491
GU	β_5	-0.065	0.483	-0.032	0.419	0.267
	SE	0.3825	0.4260	0.3185	0.3927	0.4038
	P	0.8660	0.2567	0.9200	0.2863	0.5083
	OR	0.938	1.621	0.969	1.520	1.306
	CI-	0.443	0.704	0.519	0.704	0.592
	CI+	1.984	3.736	1.808	3.282	2.883

[1]Significant effects. n_{total}: Number of investigated cases; n (+): Number of positive cases; CG: Chronic gastritis; DU: Duodenal ulcer; GU: Gastric ulcer; OR: Odds ratios; CI: Confidence intervals; *H. pylori*: *Helicobacter pylori*. Critical *P*-values were adopted after a Bonferroni correction for the number of similar tests: $P = 0.05/5 = 0.01$ for overall model and for each independent factor.

found to be a higher prevalence of *H. pylori* in DU (64%) than in GU (57%) patients[37]. These results suggest the participation of a major number of risk factors not associated with *H. pylori* involved in the development of GU rather than DU. Moreover, a very important and large scale follow-up study of ulcer patients performed in Europe[43] showed that GU can be a risk factor in the development of gastric cancer, while in DU this relationship was inversely observed, which suggests that GU and gastric cancer have etiologic factors in common that are not found in DU.

As a consequence of *H. pylori* gastric mucosa colonization, gastric acid secretion is altered with it being induced or impaired in response to the release of factors produced or induced by the bacterium, resulting in different topographic phenotypes of gastritis and the presence of atrophy. Gastritis associated with atrophy in the corpus is accompanied by hypochlorhydria and carries the highest risk for GU and cancer, whereas hyperchlorhydria produced by gastritis in the antrum predisposes to DU[43,44]. In our patients, antrum atrophy was more prevalent in DU than in GU, corroborating the hypothesis that DU can be a result of antral gastric atrophy. However, increasing age and not *H. pylori* presence was significantly related to the occurrence of atrophy and intestinal metaplasia. These results are in agreement with an epidemiological study performed in a rural population of Korea[45] and also in previous gastric physiological studies which have demonstrated the association of atrophic gastritis with increasing age[46,47].

In Northern Peninsular Malaysia, the prevalence of *H. pylori* increased in adult old-age groups, even when old and geriatric adults were compared[48]. In our investigated population, *H. pylori* incidence decreased significantly with age (Figure 4). These results can be indicative of a populational specific characteristic associated with the clearing of *H. pylori* during the course of a chronic infection, or can be related to the diagnosis of *H. pylori* in antrum biopsies since the expression of gastritis in the antrum, but not in the cardia or corpus, seems to decrease with age[49]. New research is necessary to answer these questions.

Polymorphisms in the m region of the *H. pylori vacA* gene affect the cell tropism of the toxin[50]; the m1 type of *vacA* shows toxicity toward a broader range of cells than the m2 type[51,52]. In Asia, where there is a high predominance of s1 alelle in the s region polymorphism of the *vacA* gene, the *vacA* m region mosaicism shows a variation within East Asia[53], with m1 strains being more prevalent in regions where there is a higher prevalence of gastric cancer, suggesting that m1 strains of *H. pylori* are more pathogenic. In Brazil, the involvement of *vacA* gene mosaicism with gastric diseases in adults is controversial[25-27]; in all these studies the size of investigated patient populations has been small and bacterium strains were isolated before genotyping which can cause a bias due to selection pressure by *in vitro* growth conditions. Our work was performed during a period of four years and the investigation of *vacA* m region mosaicism was done directly on biopsy specimens by PCR. Our results are the first in Brazil to find association of *vacA* m1 allele specific to adult DU patients when compared to GU and CG ($P < 0.0018$), indicating that in our region high prevalence of *H. pylori* and the strains harboring the *vacA* m1 genotype are more frequently associated with the development of DU.

Nowadays, PU remains the cause of significant morbidity, especially in older age groups, representing an important world health problem[54]. Its etiology in either the stomach (GU) or duodenum (DU) is multifactorial and depends on the interplay of a gastritis phenotype and of physiological gastroduodenal alterations as a result of environment, *H. pylori* genetic background and host interactions, which vary regionally. In this study we found that PU is associated with male gender when compared to CG and that there were risk factor differences associated with DU and GU. *H. pylori* and *vacA* m1 genotype

were associated with DU while older age at disease commitment was associated with GU. Thus, in spite of the few large scale Brazilian studies on epidemiological characteristics of gastric diseases with stratification of PU in GU and DU, we find high regional variation, indicating that local population investigation has to be carried out in order to improve treatment and prevention of severe gastric diseases.

COMMENTS

Background

Helicobacter pylori (*H. pylori*), a Gram-negative microaerophylic bacterium, is associated with a broad spectrum of digestive tract diseases such as chronic gastritis, gastric and duodenal ulcers, gastric cancer and lymphoproliferative disorder. *H. pylori* infection prevalence and clinical outcome of the colonized patients varies according to several considerations including bacterial factors and host and environmental characteristics such as age, ethnic group, genera, geography and socioeconomic conditions.

Research frontiers

The physiologic mechanisms involved in these *H. pylori*-induced pathological differences are still unknown; however, one of the major bacterium virulence factors, the vacuolating cytotoxin A (*vacA*), seems to be involved. The s1, i1, and m1 types have been shown to be independently associated with more severe forms of *H. pylori*-induced diseases.

Innovations and breakthroughs

Gastric biopsies positive for *H. pylori* by rapid urease test were investigated for *vacA* medium (m) region mosaicism by polymerase chain reaction. Logistic regression analysis was performed to verify the association of age, sex, histopathologic alterations, *H. pylori* diagnosis and *vacA* m region mosaicism.

Applications

These results can be indicative of a populational specific characteristic associated with the clearing of *H. pylori* during the course of a chronic infection, or can be related to the diagnosis of *H. pylori* in antrum biopsies since the expression of gastritis in the antrum, but not in the cardia or corpus, seems to decrease with age. New research is necessary to answer these questions.

Peer review

This is a good descriptive study in which authors analyze age, sex, histopathology and *H. pylori* status, as risk factors for gastroduodenal disease outcome in Brazilian dyspeptic patients. The results are interesting and suggest that male gender was a risk factor for peptic ulcer; ageing for gastric ulcer, atrophy and metaplasia; and *H. pylori* of *vacA* m1 genotype for duodenal ulcer.

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BRIEF ARTICLE

Argon plasma coagulation for superficial esophageal squamous-cell carcinoma in high-risk patients

Kumiko Tahara, Satoshi Tanabe, Kenji Ishido, Katsuhiko Higuchi, Tohru Sasaki, Chikatoshi Katada, Mizutomo Azuma, Kento Nakatani, Akira Naruke, Myungchul Kim, Wasaburo Koizumi

Kumiko Tahara, Satoshi Tanabe, Kenji Ishido, Katsuhiko Higuchi, Tohru Sasaki, Chikatoshi Katada, Mizutomo Azuma, Kento Nakatani, Akira Naruke, Myungchul Kim, Wasaburo Koizumi, Department of Gastroenterology, Kitasato University East Hospital, Kanagawa 252-0380, Japan

Author contributions: Tahara K and Tanabe S contributed equally to this work; Tahara K, Tanabe S, Ishido K, Higuchi K, Sasaki T, Katada C, Azuma M, Nakatani K, Naruke A, Kim M and Koizumi W designed the research and were also involved in editing the manuscript.

Correspondence to: Dr. Kumiko Tahara, Department of Gastroenterology, Kitasato University East Hospital, 2-1-1 Asamizodai, Minami-ku, Sagamihara, Kanagawa 252-0380, Japan. kumiko-t@insti.kitasato-u.ac.jp
Telephone: +81-42-7489111 Fax: +81-42-7498690
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Abstract

AIM: To evaluate the usefulness and safety of argon plasma coagulation (APC) for superficial esophageal squamous-cell carcinoma (SESC) in high-risk patients.

METHODS: We studied 17 patients (15 men and 2 women, 21 lesions) with SESC in whom endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), and open surgery were contraindicated from March 1999 through February 2009. None of the patients could tolerate prolonged EMR/ESD or open surgery because of severe concomitant disease (e.g., liver cirrhosis, cerebral infarction, or ischemic heart disease) or scar formation after EMR/ESD and chemoradiotherapy. After conventional endoscopy, an iodine stain was sprayed on the esophageal mucosa to determine the lesion margins. The lesion was then ablated by APC. We retrospectively studied the treatment time, number of APC sessions per site, complications, presence or absence of recurrence, and time to recurrence.

RESULTS: The median duration of follow-up was 36 mo (range: 6-120 mo). All of the tumors were macroscopically classified as superficial and slightly depressed type (0-Ⅱc). The preoperative depth of invasion was clinical T1a (mucosal cancer) for 19 lesions and clinical T1b (submucosal cancer) for 2. The median treatment time was 15 min (range: 10-36 min). The median number of treatment sessions per site was 2 (range: 1-4). The median hospital stay was 14 d (range: 5-68 d). Among the 17 patients (21 lesions), 2 (9.5%) had recurrence and underwent additional APC with no subsequent evidence of recurrence. There were no treatment-related complications, such as bleeding or perforation.

CONCLUSION: APC is considered to be safe and effective for the management of SESC that cannot be resected endoscopically because of underlying disease, as well as for the control of recurrence after EMR and local recurrence after chemoradiotherapy.

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Key words: Argon plasma coagulation; Superficial esophageal cancer; Squamous-cell carcinoma; High-risk patient; Endoscopic therapy

Peer reviewers: Piero Marco Fisichella, MD, Department of Surgery, Loyola University Medical Center, 2160 S. 1st Ave, Maywood, IL 60153, United States; Jan Bures, Professor, MD, PhD, Faculty of Medicine, Department of Gastroenterology, University Teaching Hospital, Charles University, Hradec Kralove 50005, Czech Republic; Satoshi Osawa, Assistant Professor, Department of Endoscopic and Photodynamic Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku 431-3192, Japan

Tahara K, Tanabe S, Ishido K, Higuchi K, Sasaki T, Katada C, Azuma M, Nakatani K, Naruke A, Kim M, Koizumi W. Argon plasma coagulation for superficial esophageal squamous-cell carcinoma in high-risk patients. *World J Gastroenterol* 2012;

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INTRODUCTION

In Japan, 329 314 persons died of malignant neoplasms in 2006; esophageal cancer was the cause of death in 11 345 persons (3.45%). Esophageal cancer is the sixth most common type of cancer in men[1]. Squamous-cell carcinoma accounts for 90% of all esophageal carcinomas. Other histologic types, including adenocarcinoma, account for approximately 5%[2]. Approximately 90% of all esophageal cancers arise in the thoracic esophagus, and the middle thoracic esophagus is the most frequent location.

Progress in endoscopic diagnostic techniques, such as iodine staining and magnifying endoscopy with narrow band imaging, has led to the detection of increased numbers of superficial carcinomas of the esophagus[3-8]. In Japan, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) are widely used as standard procedures for the treatment of superficial esophageal squamous-cell carcinoma (SESC)[9-13]. Radiofrequency ablation (RFA) is used as an endoscopic treatment for superficial esophageal cancer in Western countries, but it is not used in Japan because this procedure is not covered by the National Health Insurance system for the treatment of superficial esophageal carcinoma[14].

Several studies have reported that argon plasma coagulation (APC) is useful for the management of Barrett's esophagus[15], Barrett's esophageal cancer[16] and early gastric cancer[17,18], but few studies have evaluated APC in patients with SESC[19,20]. We retrospectively studied the safety and usefulness of APC for SESC in high-risk patients with underlying diseases that precluded EMR and ESD and in those in whom APC was performed to control local recurrence after EMR or chemoradiotherapy.

MATERIALS AND METHODS

The study group comprised 17 patients (15 men and 2 women; 21 lesions) who underwent ablation of their tumors by APC at the Department of Gastroenterology, Kitasato University East Hospital, from March 1999 through February 2009 (Table 1). The median age of the subjects was 68 years (range: 57-80 years). At the time of diagnosis, 11 patients were asymptomatic, and SESC was diagnosed incidentally on screening examinations for other diseases (peptic ulcer in 5 patients, esophageal varices in 2, gastritis in 2, after surgery for gastric cancer in 1, and after surgery for tongue cancer in 1). The other 6 patients exhibited symptoms (epigastric pain in 3, heartburn in 2, and hoarse voice in 1), and SESC was diagnosed upon further evaluation. All of the tumors were macroscopically classified as superficial and slightly depressed type (0-Ⅱc). The tumor diameter ranged from approximately 1 cm to approximately half of the circumference of the esophagus. The preoperative depth of invasion was clinical T1a (mucosal cancer) for 19 lesions and clinical T1b (submucosal cancer) for 2. Histopathologically, all of the tumors were confirmed to be esophageal squamous-cell carcinomas. EMR and ESD could not be performed because of underlying disease in 11 patients (15 lesions). APC was used to treat local recurrence after EMR or multiple metachronous tumors in 4 patients (4 lesions) and to manage local recurrence after chemoradiotherapy in 2 patients (2 lesions). Of the 17 patients, 13 (76.5%) had the following underlying diseases: liver cirrhosis in 5 patients, heart disease in 4, cerebral infarction in 1, after right lung resection with thrombophlebitis in 1, colon cancer operation in 1, and dementia in one patient. After informed consent was obtained from patients in whom anticoagulants could not be discontinued, those in whom prolonged endoscopic therapy was precluded by respiratory failure, and a patient aged 80 years, these patients asked to undergo APC among other possible treatment options. APC was therefore performed.

Table 1 Patient and lesion characteristics

Gender (men/women)	15/2
Mean age (range), yr	68 (57-80)
Location Ce/Ut/Mt/Lt/Ae	0/0/14/7/0
Macroscopic type	0-Ⅱc 21
Depth of invasion	T1a (m) 19
	T1b (sm) 2
Previous treatment	6 (35.3%) (EMR 3, ESD 1, and CRT 2)
Concomitant disease	13 (76.5%) (liver cirrhosis 5, heart disease 4, cerebral infarction 1, other 3)

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; CRT: Chemoradiotherapy.

Before APC, endoscopy was performed to evaluate the macroscopic characteristics of the tumor. Next, 1.5% Lugol's solution (a diluted iodine-potassium solution) was sprayed on the mucosa to assess the lesion margins, and a biopsy specimen was taken and examined histopathologically. Endoscopic ultrasonography (probe, UM-DP20-25R, Olympus, Tokyo, Japan) was also performed before treatment to assess the depth of invasion. The absence of metastasis was confirmed by computed tomography. All of the tumors were macroscopically classified as superficial in type according to the Guidelines for Clinical and Pathologic Studies on Carcinoma of the Esophagus by the Japanese Society for Esophageal Disease[21]. If possible, oral anticoagulants and antiplatelet agents were discontinued before treatment. APC was performed using an Olympus videoscope, a high-frequency oscillator unit (Erbotom ICC200, 1999 to 2004 or APC 2, 2004 to 2009, Elektromedizin Co. Ltd., Tuebingen, Germany), an argon gas supply unit, and a flexible argon plasma coagulator with an argon gas flow of 2 L/min and a high-frequency output of 40 W. The treatment technique was as follows. After conventional endoscopy, the lesion was stained with iodine (Figure 1A, B), and its circumference was marked by APC (Figure 1C). Subsequently, the entire

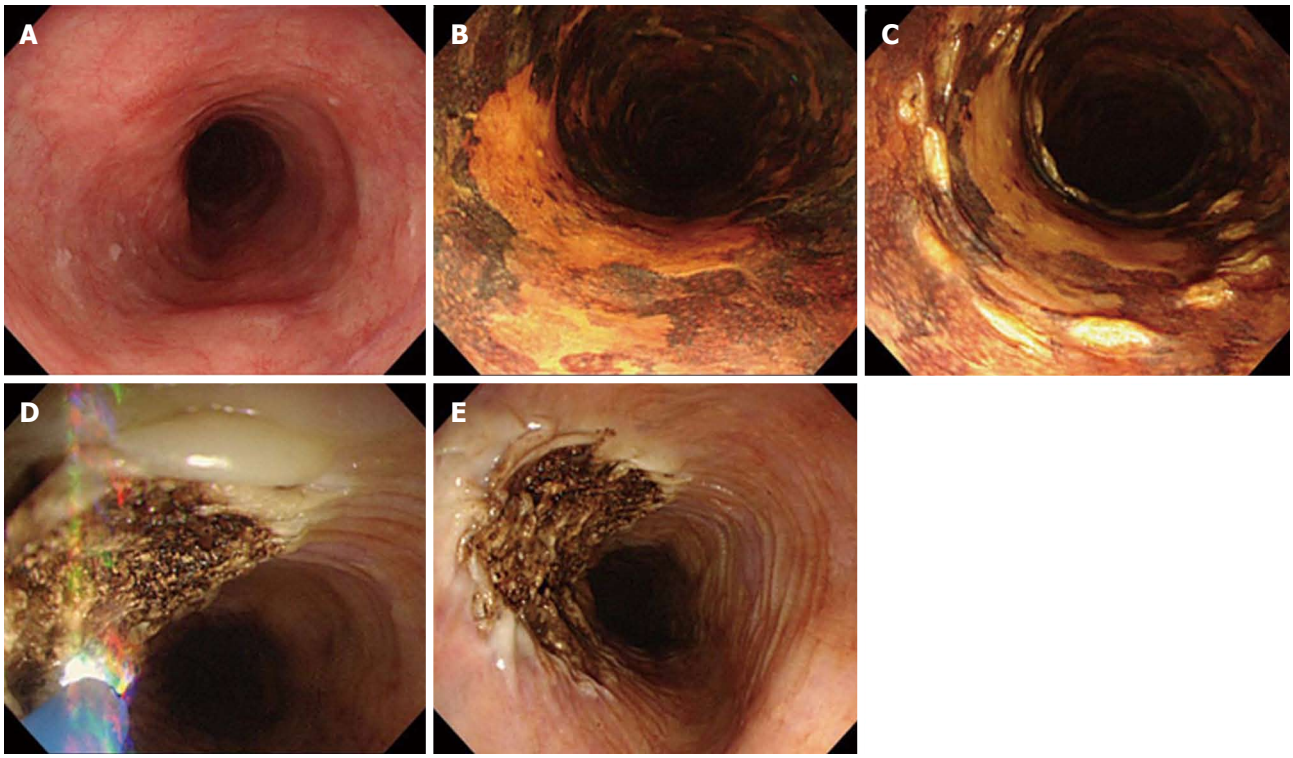


Figure 1 Treatment procedure using argon plasma coagulation. A: Conventional endoscopy; B: Chromoendoscopy with iodine staining; C: Marking by argon plasma coagulation (APC); D: Marked area was treated by APC; E: The region was completely coagulated.

Table 2 Patients characteristics

No.	Age (yr)	Sex	Depth	Time (min)	Times	Hospitalization days	Interval until recurrence (mo)	Retreatment	Illness
1	66	M	m	14	1	11	-	-	After right lung resection, thrombophlebitis[1]
2	59	M	m m	22	4	35	-	-	LC (HCV), esophageal varices
3	68	M	m	26	4	68	7	APC	LC (alcoholic), IHD, remnant stomach
4	67	M	m1	30	1	12	-	-	OMI (three vessel stenosis)[1]
5	67	M	m	15	1	53	-	-	LC (HCV), HCC, esophageal varices, DM
6	79	M	m	10	1	13	-	-	Dementia
7	66	M	sm	34	2	47	-	-	LC (alcoholic), esophageal varices, DM
8	60	M	m	16	2	14	-	-	Arrhythmia, mitral regurgitation
9	74	M	m1	14	3	11	-	-	Cerebral infarction
10	66	M	m2	36	2	17	-	-	Post tongue cancer operation
11	71	M	m1 m1 m1 m1	28	2	12	-	-	Multiple SESC
12	68	F	m1	15	1	0	-	-	Post colon cancer operation
13	76	M	m1	15	1	5	-	-	lesion of near after ESD scar
14	73	M	m1	15	1	0	-	-	Recurrence after EMR
15	70	M	m1	25	2	16	-	-	IHD[1]
16	57	M	sm1	12	4	12	3	APC	LC, recurrence after CRT
17	80	F	m2	15	2	14	-	-	Senior age, IHD, recurrence after CRT

[1]Anticoagulant therapy; m: Mucosa; sm: Submucosa; m1: Epithelium; m2: Lamina propria mucosae; sm1: Superficial layer of submucosa; APC: Argon plasma coagulation; LC: Liver cirrhosis; IHD: Ischemic heart disease; OMI: Old myocardial infarction; HCC: Hepatocellular carcinoma; DM: Diabetes mellitus; SESC: Superficial esophageal squamous-cell carcinoma; ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; CRT: Chemoradiotherapy.

lesion inside the marked area was ablated until it turned black (Figure 1D, E). If residual tumor was suspected on follow-up endoscopy 1 wk after APC, the procedure was repeated, exercising care to avoid excessive ablation. Thereafter, follow-up endoscopy was performed at approximately 2 mo, 6 mo, 12 mo, 18 mo, 24 mo, and 36

Table 3 Outcomes of endoscopic argon plasma coagulation treatment (n = 21)

Median duration of follow up (mo)	36 (6-120)
Median time required for treatment (min)	15 (10-36)
Median number of treatment sessions(times)	2 (1-4)
Median duration of hospitalization (d)	14 (5-68)
Complications	
Postoperative bleeding	0
Perforation	0
Residual tumor and recurrence	2 (9.5%)

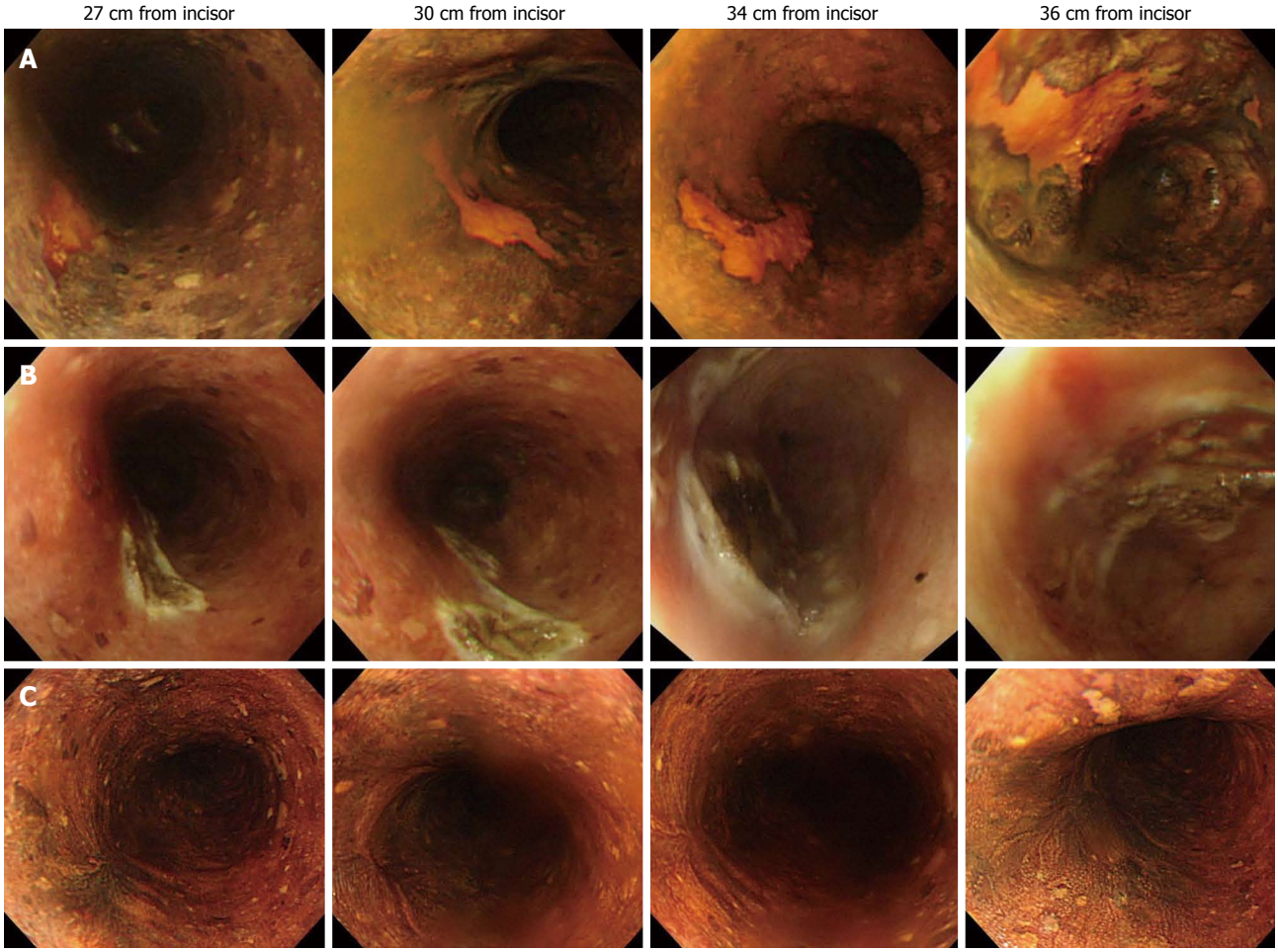


Figure 2 Case presentation: Superficial esophageal carcinoma with four lesions. A: Endoscopic findings before treatment; B: Endoscopic findings after treatment; C: Endoscopic findings 2 years after treatment.

mo. Recurrence was confirmed by endoscopic examination after the application of iodine staining and biopsy. We retrospectively studied the treatment time, the number of APC sessions per site, the presence or absence of recurrence, the time to recurrence, and complications. The median follow-up period was 36 mo (range: 6-120 mo). Informed consent was obtained from all patients in accordance with the ethical guidelines of the Declaration of Helsinki.

RESULTS

The patients' demographic characteristics are shown in Tables 1 and 2. The results of treatment and follow-up are shown in Table 3. The median time per one-treatment session was only 15 min (range: 10-36 min). The median number of treatment sessions per site was 2 (range: 1-4). There were no treatment-related complications, such as bleeding or perforation. The median duration of follow-up was 36 mo (range: 6-120 mo). Among the 21 lesions, 19 (90.0%) showed no evidence of recurrence. The 2 other lesions recurred locally 3 mo and 7 mo after APC, respectively. Both recurrences were treated by repeat APC ablation. At the time of this writing, 1 patient had died of tongue cancer and the others were alive, with no distinct evidence of metastasis or recurrence.

Case presentation

The patient was a 71-year-old man who had 4 lesions. APC was selected for treatment because prolonged ESD would have been difficult. Superficial and slightly depressed type (0-Ⅱc) lesions unstained with iodine staining were found at 4 sites, located 27 cm, 30 cm, 34 cm, and 36 cm from the incisor teeth (all of the lesions were classified as clinical T1a) (Figure 2A). APC ablation was performed at all 4 sites (Figure 2B). Follow-up endoscopy after 2 years showed no lesions *via* iodine staining and no other evidence of recurrence (Figure 2C).

DISCUSSION

APC is a non-contact technique that uses argon gas to induce tissue coagulation. In the field of gastrointestinal endoscopy, APC has been used to arrest gastrointestinal bleeding[22], ablate Barrett's esophagus[14], perform sclerotherapy of esophageal varices, and treat early gastric cancer[17,18]. At present, EMR and ESD are the endoscopic treatments of choice for superficial esophageal cancer, and studies of APC in patients with SESC are scarce[19,20].

However, EMR and ESD are associated with an increased risk of bleeding in patients with liver cirrhosis or esophageal varices and those who are receiving anticoagulant therapy[23]. Akiyama *et al*[24] used endoscopic variceal ligation for minute cancer of the esophagogastric junction accompanied by esophageal varices associated with a high risk of bleeding. ESD is thus often difficult to perform safety[25,26].

In this study, we evaluated the usefulness and safety of APC in 11 patients (15 lesions) in whom EMR was precluded by concurrent disease, 4 with recurrence after EMR, and 2 with local recurrence after chemoradiotherapy. Of the 17 patients, 13 (76.5%) had underlying diseases, such as cardiopulmonary disease and liver cirrhosis (Table 1). Two patients (9.5%) experienced tumor recurrence but received repeat APC with no subsequent recurrence. No patient experienced complications such as bleeding or perforation.

Gastrointestinal perforation caused by APC has been reported[27,28]. Grund *et al*[29] performed APC in a large series of patients and reported that the incidences of gastrointestinal perforation and intestinal emphysema were 0.25% and 0.46%, respectively. The possibility of complications should thus be borne in mind when APC ablation is performed.

Patients with recurrence after EMR or local recurrence after chemoradiotherapy have scars caused by prior treatment, increasing the risk of perforation on repeat EMR. Nomura *et al*[30] used APC to control local recurrence after chemoradiotherapy and recurrence after EMR. Both conditions could be treated safely within a short time. Two of 10 patients experienced recurrence, but APC exhibited a low risk of perforation and allowed oral intake to be resumed earlier than EMR. APC was therefore recommended for patients in whom EMR is difficult to perform. In our study, APC was completed safely and within a short time.

Photodynamic therapy is another option for the endoscopic treatment of superficial esophageal cancer but requires pretreatment and light shielding after application[31,32]. In contrast, APC is more convenient and straightforward, with a simple treatment procedure.

In conclusion, our results showed that APC effectively ablated SESC in patients with underlying disease and in those in whom EMR and ESD were not feasible. By repeating APC as required, the tumors were reliably ablated. APC is thus considered to be a safe and effective treatment that can be accomplished within a relatively short time. To confirm our results, additional prospective studies of larger numbers of patients are needed.

COMMENTS

Background

In Japan, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) are standard treatments for superficial esophageal squamous-cell carcinoma (SESC). However, the use of these procedures is contraindicated by underlying disease in certain patients.

Research frontiers

Argon plasma coagulation (APC) is useful for the management of Barrett's esophageal cancer and early gastric cancer, but few studies have evaluated APC in patients with SESC.

Innovations and breakthroughs

APC effectively ablated SESC in patients with underlying disease, as well as in those for whom EMR and ESD were not feasible.

Applications

APC is considered to be safe and effective for the management of superficial carcinoma of the esophagus that cannot be resected endoscopically because of underlying disease, as well as for the control of recurrence after EMR and local recurrence after chemoradiotherapy.

Peer review

In this study, the authors performed retrospective study that assessed the clinical usefulness and safety of APC for SESC in high risk patient. This study showed APC would be one of the good option to treat SESC especially in high risk patient, although further prospective study in large sample size is necessary to conclude it.

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BRIEF ARTICLE

Hepatitis B virus pre-S2 start codon mutations in Indonesian liver disease patients

Andi Utama, Marlinang Diarta Siburian, Ismail Fanany, Mariana Destila Bayu Intan, Rama Dhenni, Tri Shinta Kurniasih, Syafruddin AR Lelosutan, Wenny Astuti Achwan, Nasrul Zubir, Arnelis, Benyamin Lukito, Irawan Yusuf, Laurentius Adrianus Lesmana, Ali Sulaiman

Andi Utama, Marlinang Diarta Siburian, Ismail Fanany, Mariana Destila Bayu Intan, Rama Dhenni, Tri Shinta Kurniasih, Molecular Epidemiology Division, Mochtar Riady Institute for Nanotechnology, Universitas Pelita Harapan, Lippo Karawaci, Tangerang 15810, Banten, Indonesia
Syafruddin AR Lelosutan, Gastroentero-Hepatology Division, Department of Internal Medicine, Gatot Soebroto Hospital, Jakarta 10410, Indonesia
Wenny Astuti Achwan, Department of Internal Medicine, Mataram General Hospital, Mataram 83127, Indonesia
Nasrul Zubir, Arnelis, Gastroentero-Hepatology Division, Department of Internal Medicine, M. Djamil Hospital, Padang 25127, Indonesia
Benyamin Lukito, Department of Internal Medicine, Siloam Hospital Lippo Karawaci, Tangerang 15810, Banten, Indonesia
Irawan Yusuf, Faculty of Medicine, Hasanuddin University, Makassar 90245, Indonesia
Laurentius Adrianus Lesmana, Ali Sulaiman, Hepatology Division, Department of Internal Medicine, Faculty of Medicine, University of Indonesia, Jakarta 10430, Indonesia
Author contributions: Utama A designed the study and prepared the manuscript; Siburian MD, Fanany I, Intan MDB, and Kurniasih TS performed the experiments; Dhenni R performed sequence and statistic analysis; Lelosutan SAR, Achwan WA, Arnelis, Lukito B, Yusuf I, Zubir N, Lesmana LA, and Sulaiman A coordinated and provided the collection of human materials and were involved in editing the manuscript.
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Correspondence to: Andi Utama, PhD, Molecular Epidemiology Division, Mochtar Riady Institute for Nanotechnology, Universitas Pelita Harapan, Jalan Boulevard Jend. Sudirman 1688, Lippo Karawaci, Tangerang 15810, Banten, Indonesia. autama@mrinstitute.org
Telephone: +62-21-54210123 Fax: +62-21-54210110
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Abstract

AIM: To identify the prevalence of pre-S2 start codon mutations and to assess their association with liver disease progression.

METHODS: The mutations were identified by direct sequencing from 73 asymptomatic carriers, 66 chronic hepatitis (CH), 66 liver cirrhosis (LC) and 63 hepatocellular carcinoma (HCC) patients. Statistical significances were determined using Fisher's exact test, χ^2 test, and *t*-test analyses whenever appropriate. Pre-S mutation as a risk factor for advanced liver disease was estimated by unconditional logistic regression model adjusted with age, sex, and hepatitis B e antigen (HBeAg). $P < 0.05$ was considered significant.

RESULTS: Mutation of the hepatitis B virus (HBV) pre-S2 start codon was found in 59 samples from 268 subjects (22.0%), with higher prevalence in patients with cirrhosis 27/66 (40.9%) followed by HCC 18/63 (28.6%), chronic hepatitis 12/66 (18.2%) and asymptomatic carriers 2/73 (2.7%) ($P < 0.001$). Logistic regression analysis showed that pre-S2 start codon mutation was an independent factor for progressive liver disease. Other mutations, at T130, Q132, and A138, were also associated with LC and HCC, although this was not statistically significant when adjusted for age, sex, and HBeAg. The prevalence of pre-S2 start codon mutation was higher in HBV/B than in HBV/C (23.0% *vs* 19.1%), whilst the prevalence of T130, Q132, and A138 mutation was higher in HBV/C than in HBV/B. The prevalence of pre-S2 start codon mutation was higher in LC (38.9%) and HCC (40.0%) than CH (5.6%) in HBeAg(+) group, but it was similar between CH, LC and HCC in HBeAg(-) group.

CONCLUSION: Pre-S2 start codon mutation was higher in Indonesian patients compared to other Asian countries, and its prevalence was associated with advanced liver disease, particularly in HBeAg(+) patients.

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Key words: Hepatitis B virus; Pre-S2 start codon; Liver disease; Hepatitis B e antigen seroconversion; Indonesia

Peer reviewers: Dr. Juan Ramón Larrubia, Gastroenterology Unit and Liver Research Unit, Guadalajara University Hospital, University of Alcalá, Donante de Sangre s/n, 19002 Guadalajara, Spain; Dr. Eric WC Tse, Department of Medicine, Queen Mary Hospital, Hong Kong, China

Utama A, Siburian MD, Fanany I, Intan MDB, Dhenni R, Kurniasih TS, Lelosutan SAR, Achwan WA, Zubir N, Arnelis, Lukito B, Yusuf I, Lesmana LA, Sulaiman A. Hepatitis B virus pre-S2 start codon mutations in Indonesian liver disease patients. *World J Gastroenterol* 2012; 18(38): 5418-5426 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i38/5418.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i38.5418

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most important infectious diseases worldwide. It is the major cause of chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC). About 40% of the world's population has had contact with, or are carriers of, HBV and it is estimated that more than 350 million people world wide have chronic liver infection[1]. Indonesia has a moderate to high endemnicity of HBV infection[2], perhaps due to the lack of proper health facilities, poor economical status, less public awareness, or incomplete vaccination.

HBV is a relaxed circular, partially double stranded 3.2 kb DNA virus[3]. It has four overlapping reading frames that encode Polymerase, Core, X and Envelope proteins[4]. The Envelope proteins are produced from a single open reading frame with three different translation sites, pre-S1, pre-S2, and S. It produces three forms of HBV surface proteins (HBs), which are the large (L), middle (M) and small (S) HBs[5]. The major component of the Envelope protein is S protein which consists of 226 amino acids and drives particle budding. The M protein is composed of the S protein with an additional of 55 amino acids termed pre-S2 attached to the N-terminus. The L protein is the M protein with an additional 108 or 119 genotype-dependent amino acids attached to the N-terminus[6].

The pre-S1 and pre-S2 region is the region with the most variability in the HBV genome[7]. This variability is in the form of deletions, insertions, or synonymous or non-synonymous nucleotide substitutions. The pre-S1 and pre-S2 regions encode the T- and B-cell epitopes which play roles in allowing neutralizing antibodies to bind, and consequent immune protection[8,9]. Therefore, HBV pre-S mutant variants may emerge as a result of selective immune pressure.

The high prevalence of HBV pre-S mutations in Asia, including pre-S deletion and pre-S2 start codon mutations, has been reported, and it has been demonstrated that these mutations were associated with progressive liver disease[10]. In addition, amino acid substitution from Phenylalanine to Lysine at codon 141 in pre-S2 region (F141L) is also associated with HCC in patients infected with HBV genotype C[11]. Furthermore, study of hepatitis B e antigen (HBeAg) (-) patients in China revealed that pre-S deletions alone, or in combination with mutations in precore and basal core promoter (BCP) of HBV, are also associated with advanced liver disease[12].

Studies on the prevalence of HBV genotypes, BCP and precore mutations, and their association with severity of liver disease in Indonesia have previously been reported[13-15]. We have recently published a molecular epidemiological study on the prevalence of pre-S deletion mutation in Indonesian subjects[16], but this paper did not describe the incidence of synonymous and non-synonymous mutations in the pre-S region. Therefore, we extended this study to investigate the prevalence of pre-S2 start codon mutation and its association with severe liver disease in Indonesian patients. In addition, other mutations in the pre-S2 regions were analyzed.

MATERIALS AND METHODS

Samples

Blood samples were obtained from 270 HBV carriers. Two subjects with genotype D were excluded due to its relatively low prevalence in the collective samples and also in Asian population in general. A total of 268 subjects were included in the study: 73 asymptomatic carriers (AC) and 195 liver disease patients which composed of 66 CH, 66 LC, and 63 HCC. Asymptomatic carrier samples were taken from donors with positive HBsAg from the Blood Transfusion Unit (Red Cross Makassar, South Sulawesi, Indonesia), between February and August 2007. Chronic hepatitis samples were from patients positive for HBsAg for more than six months, and who had more than twice the normal alanine aminotransferase (ALT) level. Liver cirrhosis was diagnosed by liver function tests and ultrasonography, whilst the diagnosis of HCC was either on the basis of ultrasonography as well as an elevated serum-fetoprotein (AFP) level (≥ 200 ng/mL), or by needle aspiration liver biopsy for samples in which the AFP level was low. Samples from CH, LC, and HCC patients were collected from Cipto Mangunkusumo Hospital, Gatot Soebroto Hospital, Klinik Hati, Jakarta, Siloam Hospital Lippo Karawaci, Tangerang, Mataram General Hospital, Mataram, Wahidin Sudirohusodo Hospital, Makassar, and M. Djamil Hospital, Padang, from May 2006 to February 2010. None of the samples had coinfection with hepatitis C virus. The HBsAg test for all samples was performed using a commercially available enzyme-linked immunosorbent assay, kit (Abbott Laboratories, Chicago, IL, United States). The HBeAg status of samples was tested by using rapid test (Intec, Xiamen, China) as described previously[16]. Blood samples were separated into plasma and stored at -70 ℃ until use. The study was

Table 1 Demographic data and the prevalence of pre-S mutations associated with progressive liver disease

	AC	CH	LC	HCC	All	*P* value (all)	*P* value (AC-CH *vs* LC)	*P* value (AC-CH *vs* HCC)
n (%)	73 (27.2)	66 (24.6)	66 (24.6)	63 (23.5)	268 (100.0)	-	-	-
Gender, *n* (%)								
Male	69 (94.5)	49 (74.2)	46 (69.7)	49 (77.7)	213 (79.5)	0.002	0.027	0.314
Female	4 (5.5)	17 (25.8)	20 (30.3)	14 (22.2)	55 (20.5)			
Age (yr, mean ± SD)	32.3 ± 9.1	41.9 ± 13.3	50.1 ± 10.9	48.6 ± 11.6	42.9 ± 13.3	< 0.001	< 0.001	< 0.001
Genotype, *n* (%)								
B	56 (76.7)	46 (69.7)	46 (69.7)	52 (82.5)	200 (74.6)	0.267	0.453	0.393
C	17 (23.3)	20 (30.3)	20 (30.3)	11 (17.5)	68 (25.4)			
HBeAg[1]								
HBeAg(+)	13 (22.0)	36 (56.3)	18 (30.5)	15 (28.3)	82 (34.9)	< 0.001	0.222	0.144
HBeAg(-)	46 (78.0)	28 (43.8)	41 (69.5)	38 (71.7)	153 (65.1)			
Mutation								
M120	2 (2.7)	12 (18.2)	27 (40.9)	18 (28.6)	59 (22.0)	< 0.001	< 0.001	0.002
T130	8 (11.0)	19 (28.8)	18 (27.3)	25 (39.7)	70 (26.1)	0.0018	0.311	0.054
Q132	11 (15.1)	19 (28.8)	21 (31.8)	26 (41.3)	77 (28.7)	0.008	0.111	0.041
A138	10 (13.7)	21 (31.8)	22 (33.3)	26 (41.3)	79 (29.5)	0.003	0.089	0.094

[1]Number of samples for HBeAg. *n* (all) = 235, *n* (AC:CH:LC:HCC) = 59:64:59:53. AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen.

approved by the Committee on Health Research Ethics of the Mochtar Riady Institute for Nanotechnology and informed consent was obtained from each subject.

Viral DNA extraction, polymerase chain reaction and sequencing

HBV DNA was extracted from 200 μL plasma using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and 80 μL of eluted DNA was stored at -70 ℃ until use. Pre-S region was amplified by nested polymerase chain reaction (PCR) using PCR Core System (Promega, Madison, WI, United States) and two sets of primers as previously described[16]. The first round was performed with primers PS1 (5'-GGGTCACCTTATTCTTGGGA-3', position 2814-2833) and PS2 (5'-CCCCGCCTGTAACACGAGCA-3', position 208-189). For the second round, primers PS3 (5'-TTGGGAACAAGATCTACAGC-3', position 2828-2847) and PS4 (5'-GTCCTGATGCGATGTTCTCC-3', position 176-157) were used. First and second round PCR were performed with the same profile for 36 cycles of 94 ℃ for 1 min, 58 ℃ for 30 s and 72 ℃ for 1 min in a thermal cycler. The PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States), directly sequenced employing an ABI 3130xl Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA, United States) with the Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Inc.) using primers PS3 and PS4[13-16].

Hepatitis B virus genotyping and pre-S mutations analysis

The HBV genotype was determined by phylogenetic analysis of the pre-S sequences. The sequences were compared to 74 HBV reference strains of eight HBV genotypes (A-H) from GenBank. Alignment and phylogenetic trees were constructed using molecular evolutionary genetics analysis 4 software (Center for Evolutionary Functional Genomics, Tempe, AZ, United States)[17]. Pre-S amino acid sequences were aligned and compared between each group to detect the mutations.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 software for Windows (SPSS Inc., Chicago, IL, United States). Statistical significances were determined using Fisher's exact test, χ^2 test, and *t*-test analyses whenever appropriate. The odds ratio and 95% CI were estimated by unconditional logistic regression model to evaluate the association of Pre-S mutation as a risk factor for advanced liver disease adjusted with age, sex and HBeAg. $P < 0.05$ was considered to be statistically significant.

RESULTS

Demographic data of subjects

Demographic data of subjects are shown in Table 1. A total of 268 subjects comprising 73 AC, 66 CH, 66 LC and 63 HCC were included in the study. The male/female ratio was significantly different among the groups ($P = 0.002$). The mean of ages for AC, CH, LC, and HCC was 32.3 ± 9.1, 41.9 ± 13.3, 50.1 ± 10.9 and 48.6 ± 11.6 years, respectively, and it was significantly different among the group ($P < 0.001$). Ratio of HBV/B to HBV/C showed no significant difference among the groups ($P = 0.267$). Of 268 subjects, 235 subjects were available for HBeAg rapid test analysis. Of 235 subjects, 82 (34.9%) had HBeAg(+) and 153 (65.1%) had HBeAg(-) and the distribution was significantly different among the group ($P < 0.001$).

Prevalence of pre-S2 start codon mutation and its association with the risk of advanced liver disease

Pre-S2 start codon mutations were found in 22.0% (59/268) of the total samples (Table 1). The mutation

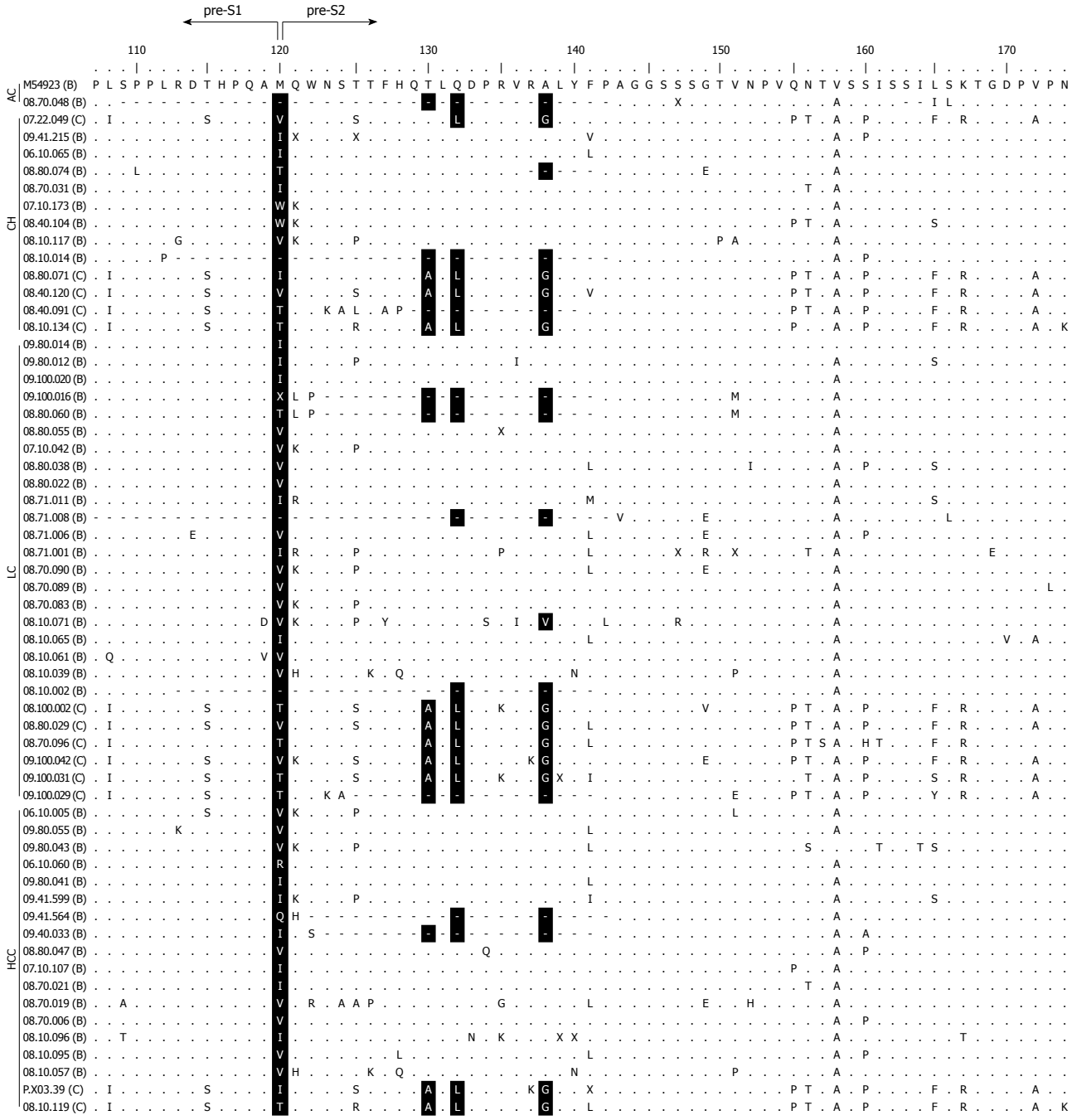


Figure 1 Amino acid alignment of 59 hepatitis B virus sequences harboring pre-S2 start codon mutation with wild type reference M54923 (genotype B). Positions of mutations are shown in black shades (M120, T130, Q132, and A138). Dots represent identical amino acids to the consensus sequence. Dashes represent deletion mutation. The names of the samples are indicated with each respective genotype in parentheses. AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver Cirrhosis; HCC: Hepatocellular carcinoma.

(M120) was either an amino acid substitution from Methionine to other amino acids or a deletion mutation (Figure 1). The prevalence of this mutation was significantly different among the groups ($P < 0.001$), and was increasingly common as disease progressed from AC (2.7%) to CH (18.2%) and LC (40.9%), but was less common in those with HCC (28.6%) than with LC but this difference was not statistically significant (Figure 2A). Multivariate regression analysis (based on 235 subjects with complete HBeAg) adjusted with age, gender and HBeAg demonstrated that M120 mutation was an independent factor in the development of progressive liver disease [OR 3.996 (1.830-8.729), $P = 0.0005$] (Table 2). These results implied that this mutation was associated with progressive liver disease and more particularly with LC than HCC.

Prevalence of other mutations in the pre-S2 region

Beside the pre-S2 start codon (M120) mutation, other mutations in the pre-S region were also analyzed. It was found that the prevalence of three amino acid changes in

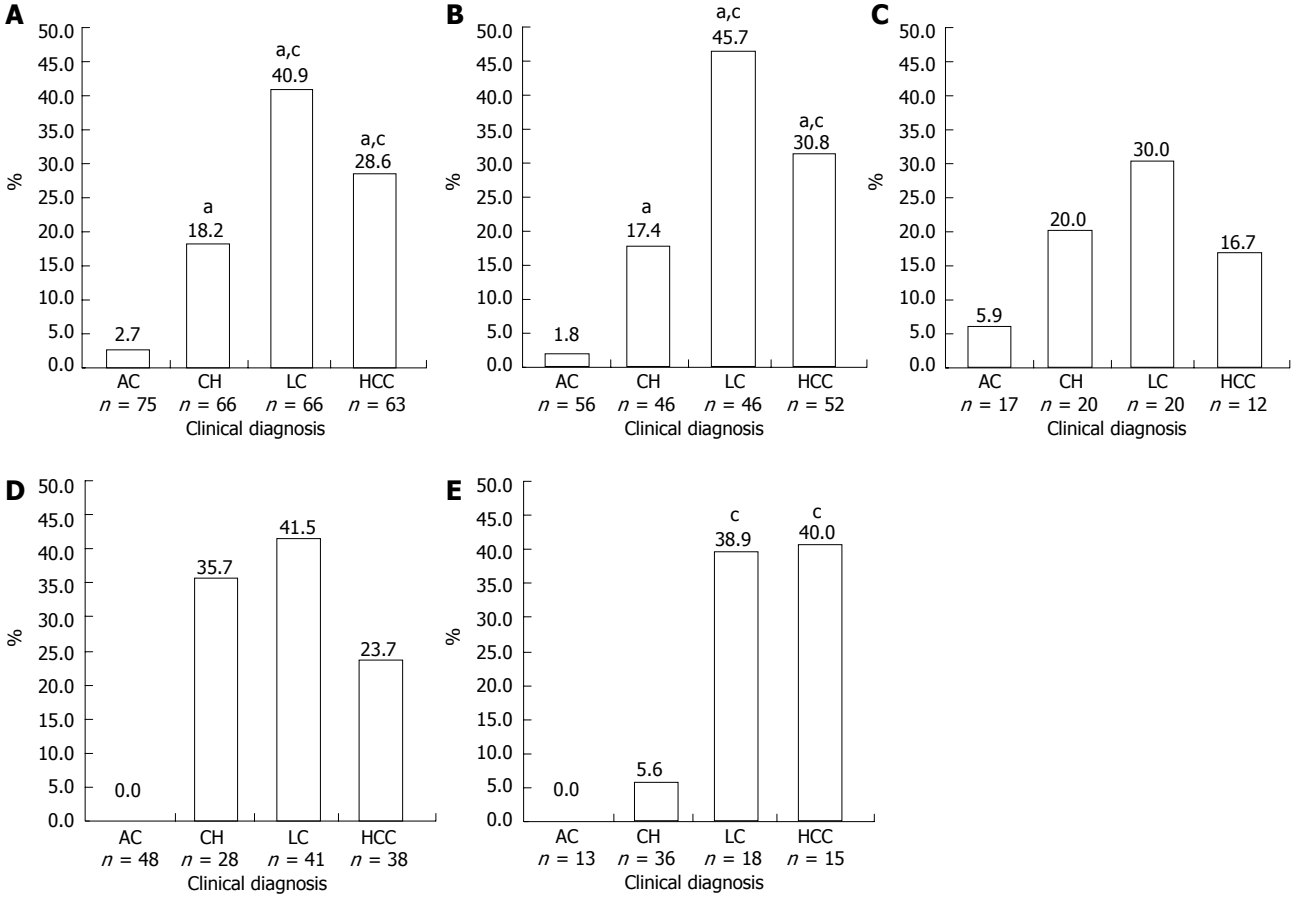


Figure 2 Prevalence of pre-S2 start codon mutation in all samples and in patients infected with hepatitis B virus/B, hepatitis B virus/C, hepatitis B e antigen (+), hepatitis B e antigen (-). A: Prevalence of pre-S2 start codon mutation in all samples; B: Prevalence of pre-S2 start codon mutation in patients infected with hepatitis B virus (HBV)/B; C: Prevalence of pre-S2 start codon mutation in patients infected with HBV/C; D: Prevalence of pre-S2 start codon mutation in patients with hepatitis B e antigen (HBeAg) (-); E: Prevalence of pre-S2 start codon mutation in patients with HBeAg(+). $^{a}P < 0.05$ *vs* AC; $^{c}P < 0.05$ *vs* CH. AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

the pre-S2 region showed significant differences among the groups (Table 1). Amino acid substitution from Threonine to other amino acids or deletion at codon 130 (T130) was detected in 26.1% of the samples, and the percentage increased in more severe liver disease ($P = 0.002$). Changes from Glutamine to other amino acids, or deletion at codon 132 (Q132), was found in 28.7% of the samples, and the prevalence was also increased with the severity of liver disease ($P = 0.008$). Similarly, amino acid substitution or deletion at Alanine 138 (A138) was found in 29.5% of samples and was highly prevalent in advanced liver disease ($P = 0.003$). Multivariate regression analysis showed that none of the mutations had significant association with progressive liver disease (Table 2).

Comparison of pre-S mutations in hepatitis B virus genotypes B and C

The samples were grouped into HBV/B and HBV/C and there were no significant differences in male/female ratios, mean age, or pattern of clinical diagnoses between the two groups (data not shown). Pre-S2 start codon mutation (M120) was more prevalent in HBV/B (23.0%) than in HBV/C (19.1%) (Table 3). In HBV/B, M120 mutation was associated with severity of liver disease ($P < 0.001$), but no association was found in HBV/C. The highest prevalence of M120 mutation in HBV/B was found in the LC group (45.7%), which was higher than that in the HCC group (30.8%), although not statistically significant (Figure 2B). Two other mutations in pre-S2 region (T130 and A138) showed significant association with the progress of liver disease in HBV/B ($P = 0.017$ and $P = 0.042$, respectively). In HBV/C, there was no association of the M120 mutation or the other mutations with progressive liver disease (Table 3, Figure 2C).

Comparison of pre-S mutations in HBeAg(-) and in HBeAg(+) patients

As shown in Table 4, the HBeAg(-) and HBeAg(+) groups differed significantly in the ratio of males to females, mean age, and pattern of clinical diagnoses. More male subjects were found in the HBeAg(-) group than in the HBeAg(+) group (83.0% *vs* 70.7%, $P = 0.035$). The mean age of subjects in the HBeAg(-) was greater than in HBeAg(+) group (44.7 ± 13.9 years *vs* 39.2 ± 11.9 years, $P = 0.003$). The prevalence of M120 mutations was higher in the HBeAg(-) group than in the HBeAg(+) group (23.5% *vs* 18.3%) (Table 5). The prevalence of this mutation was significantly different among the samples

Table 2 Pre-S mutation as risk factor for progressive liver disease adjusted with age, sex, and hepatitis B e antigen (n = 235)

	P-value	OR (95% CI)	*P*-value	OR adjusted (95% CI)
AC-CH *vs* LC-HCC				
Age, mean ± SD	1.99E-11	1.099 (1.069-1.130)		
Gender (male)	0.049	0.531 (0.281-1.002)		
HBeAg(-)	0.096	1.585 (0.921-2.730)		
Mutation				
M120	3.23E-06	4.942 (2.427-10.064)	0.0005	3.996 (1.830-8.729)
T130	0.078	1.684 (0.940-3.016)	0.531	1.246 (0.626-2.483)
Q132	0.029	1.885 (1.064-3.339)	0.352	1.379 (0.701-2.712)
A138	0.041	1.801 (1.020-3.178)	0.580	1.209 (0.617-2.370)
AC-CH *vs* LC				
Age, mean ± SD	8.86E-09	1.096 (1.063-1.131)		
Gender (male)	0.027	0.442 (0.213-0.920)		
HBeAg(-)	0.222	1.508 (0.778-2.922)		
Mutation				
M120	9.50E-07	6.343 (2.878-13.980)	4.00E-05	6.406 (2.640-15.540)
T130	0.311	1.440 (0.710-2.918)	0.672	1.198 (0.518-2.770)
Q132	0.111	1.740 (0.878-3.449)	0.364	1.457 (0.646-3.287)
A138	0.089	1.791 (0.911-3.523)	0.485	1.330 (0.597-2.964)
AC-CH *vs* HCC				
Age, mean ± SD	2.13E-07	1.084 (1.052-1.118)		
Gender (male)	0.314	0.663 (0.298-1.479)		
HBeAg(-)	0.144	1.677 (0.835-3.372)		
Mutation				
M120	0.002	3.651 (1.570-8.490)	0.052	2.538 (0.992-6.494)
T130	0.054	1.987 (0.981-4.022)	0.402	1.413 (0.629-3.174)
Q132	0.041	2.056 (1.024-4.129)	0.498	1.321 (0.590-2.953)
A138	0.094	1.811 (0.900-3.644)	0.744	1.144 (0.509-2.571)

AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; CI: Confidence interval; OR: Odds ratio; HBeAg: Hepatitis B e antigen.

Table 3 Prevalence of pre-S mutations in hepatitis B virus genotype B and C associated with progressive liver disease

Mutations	Genotype B						Genotype C					
	AC n = 56	CH n = 46	LC n = 46	HCC n = 52	All n = 200	*P*-value	AC n = 17	CH n = 20	LC n = 20	HCC n = 12	All n = 68	*P*-value
M120	1 (1.8)	8 (17.4)	21 (45.7)	16 (30.8)	46 (23.0)	< 0.001	1 (5.9)	4 (20.0)	6 (30.0)	2 (16.7)	13 (19.1)	0.324
T130	6 (10.7)	11 (23.9)	10 (21.7)	19 (36.5)	46 (23.0)	0.017	2 (11.8)	8 (40.0)	8 (40.0)	6 (50.0)	24 (35.3)	0.098
Q132	9 (16.1)	11 (23.9)	12 (26.1)	20 (38.5)	52 (26.0)	0.067	2 (11.8)	8 (40.0)	9 (45.0)	6 (50.0)	25 (36.8)	0.081
A138	8 (14.3)	12 (26.1)	13 (28.3)	20 (38.5)	53 (26.5)	0.042	2 (11.8)	9 (45.0)	9 (45.0)	6 (50.0)	26 (38.2)	0.070

AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

with different clinical diagnoses both in HBeAg(-) and HBeAg(+) patients. In the HBeAg(-) group, the highest prevalence of M120 mutation was found in LC (41.5%), followed by CH (35.7%) and HCC (23.7%), and no mutations were found in the AC group. However, no statistically significant differences were found between CH, LC and HCC (Figure 2D). Among HBeAg(+) patients, the highest frequency of M120 mutation was detected in those with HCC (40.0%), followed by those with LC (38.9%), and CH (5.6%), and no mutations were found in the AC group. Significant differences were found between those with CH and LC and CH and HCC, but not between those with LC and HCC (Figure 2E). On the other hand, no significant correlation of T130, Q132 and A138 mutations with severity of liver disease was found in either the HBeAg(+) or HBeAg(-) groups (Table 5).

DISCUSSION

Many studies have been reported on the association of pre-S mutations with severity of liver disease. Studies from Taiwan and China demonstrated that the pre-S deletion mutation is associated with HCC[18-20]. In addition, the pre-S2 deletion mutation was also specifically associated with increased risk of HCC in Asian children[21]. Other studies also reported that pre-S deletion and pre-S2 start codon mutations was found to be significantly associated with HCC[22,23]. A meta analysis study including 43 reports found that mutations at the promoter sites of pre-S1 and pre-S2 are significantly associated with an increased risk of HCC[24]. However, the diagnostic or predictive value of those mutations for HCC is limited because the frequencies of these mutations in the pa-

Table 4 Demographic data of samples with hepatitis B e antigen (+) and hepatitis B e antigen (-) *n* (%)

	HBeAg(+)	HBeAg(-)	*P*-value
	82 (34.9)	153 (65.1)	
Gender,			
Male	58 (70.7)	127 (83.0)	0.028
Female	24 (29.3)	26 (17.0)	
Age, mean ± SD	39.2 ± 11.9	44.7 ± 13.9	0.003
Genotype,			
B	56 (68.3)	116 (75.8)	0.215
C	26 (31.7)	37 (24.2)	
Clinical diagnosis,			
AC	13 (15.9)	46 (30.1)	< 0.001
CH	36 (43.9)	28 (18.3)	
LC	18 (22.0)	41 (26.8)	
HCC	15 (18.3)	38 (24.8)	

AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen.

tients with HCC were low; pre-S1 and pre-S2 promoter mutations were 19.7% and 15.3%, respectively[24].

Recently, we reported on the prevalence of pre-S deletion mutation in Indonesian HBV carriers. The prevalence of pre-S deletion was 12.1% which is considered low compared to previous reports from other populations[16]. In the present study, which extends the analysis of this group of patients, pre-S2 start codon mutations were found in 22.0% of the samples. In Indonesia, it therefore appears that the pre-S2 start codon mutation is a more common type of mutation compared to the pre-S deletion mutation. The prevalence of pre-S2 start codon mutations was higher in the cirrhosis group than the HCC group, which is in accordance with the previous report[22]. Both pre-S2 deletion and start codon mutations cause alterations or loss of the M-protein, which has the T- and B-cell epitopes. These altered M-proteins can stimulate hepatocyte proliferation, and cause the formation of type II ground glass hepatocytes[25,26]. In this study, it was found that this mutation is more prevalent in the liver cirrhosis group, which was older than the other groups, indicating prolonged infection by HBV in this group. It has been suggested that pre-S2 deletion mutations, which affect the T-cell epitope and hence the ability of the virus to be neutralized, represent mutants that are selected under immune pressure, and are thus able to persist during chronic HBV infection[26]. Pre-S2 start codon mutations may have the same effect, since the mutation prevents production of M-protein. Furthermore, it has been reported that the loss of M-protein does not affect virus infectivity, but instead it may function as a spacer that supports conformational changes of L protein, or as a subdomain of the L protein in virus entry[6]. In this way it might allow mutants that lack M protein to emerge without affecting the virus life cycle.

Other mutations in the pre-S2 region were shown to be significantly associated with progressive liver disease. However regression analysis showed these amino acid changes or deletion at three codon sites, T130, Q132, and A138 were not statistically significantly associated with the increased risk of cirrhosis and HCC. The prevalence of these three mutations was considerably higher in the HCC group (38.5%-40.0%). Few studies on amino acid substitutions in the pre-S2 region and their association with progressive liver disease have been reported. An amino acid change from Glycine to Arginine at pre-S2 at codon 149 (G149R) was found in 52.9% of chronic carriers with spontaneous clearance of HBV surface antigen[27]. More recently it was found that a mutation at position 141 (F141L mutation) was associated with increased risk of HCC in patients with HBV/C infections[11]. However the mutation found in this study did not correlate with coexistence of HBsAg and anti-HBs, but may alter the B- and T-cell epitopes of the S protein, and thereby increase the risk of cirrhosis and HCC by decreasing immune recognition.

In the present study, the prevalence of pre-S2 start codon mutation was higher in HBV/B than HBV/C, although the difference was not statistically significant. In addition, a significant association of pre-S2 start codon mutation with severe liver disease was found in HBV/B, while there was no significant difference in the frequency of this mutation in HBV/C. A study in Thailand revealed a higher prevalence of pre-S2 deletions and preS2 start codon mutation in HBV/C than in HBV/B[28]. Another recent report showed a higher prevalence of pre-S2 deletions in HBV/C and this was associated with HCC in children[29]. The differences between previous studies and the present study are probably due to the difference in percentage of HBV/C samples in subjects included in the studies (the number of HBV/C infections was lower in the present study). On the other hand, in the present study, the prevalence of the three other mutations in the pre-S2 region (T130, Q132, and A138) was higher in those with HBV/C than those with HBV/B. In those with HBV/B, the incidence of T130 and A138 mutations was significantly different between the disease groups, and was increased with advanced liver disease (Table 3). However, the prevalence of these mutations was not significantly different between disease groups with HBV/C, possibly due to the low number of HBV/C samples included in this study.

The prevalence of pre-S2 start codon mutations was higher in HBeAg(-) samples than in HBeAg(+) samples (23.5% *vs* 18.3%), but interestingly in the HCC group the prevalence of this mutation was higher in HBeAg(+) samples than in HBeAg(-) samples (40.0% *vs* 23.7%) (Table 5). No mutations were found in asymptomatic carriers of either group. The prevalence of mutations in the HBeAg(+) group showed significant association between chronic hepatitis and cirrhosis as well as HCC, while in the HBeAg(-) group there were no differences found among CH, LC and HCC. Previous studies have described the roles of pre-S deletions on the progression of liver disease in HBeAg(-) patients in longitudinal study, and found that pre-S deletions were significantly associated with the development of liver cirrhosis[12]. In

Table 5 Prevalence of pre-S mutations in hepatitis B e antigen (-) and hepatitis B e antigen (+) associated with progressive liver disease

Mutation	HBeAg(-)						HBeAg(+)					
	AC *n* = 46	CH *n* = 28	LC *n* = 41	HCC *n* = 38	All *n* = 155	*P*-value	AC *n* = 13	CH *n* = 36	LC *n* = 18	HCC *n* = 15	All *n* = 82	*P*-value
M120	0 (0.0)	10 (35.7)	17 (41.5)	9 (23.7)	36 (23.5)	< 0.001	0 (0.0)	2 (5.6)	7 (38.9)	6 (40.0)	15 (18.3)	< 0.001
T130	6 (13.0)	10 (35.7)	11 (26.8)	13 (34.2)	40 (26.1)	0.082	2 (15.4)	9 (25.0)	6 (33.3)	6 (40.0)	23 (28.0)	0.474
Q132	6 (13.0)	10 (35.7)	12 (29.3)	14 (36.8)	42 (27.5)	0.057	3 (23.1)	9 (25.0)	8 (44.4)	6 (40.0)	26 (31.7)	0.389
A138	6 (13.0)	10 (35.7)	13 (31.7)	13 (34.2)	42 (27.5)	0.072	2 (15.4)	11 (30.6)	8 (44.4)	6 (40.0)	27 (32.9)	0.344

AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen.

another longitudinal study observing the natural course of HBV there was no clear relationship between HBeAg seroconversion and pre-S deletions[30]. The present study was a cross-sectional study which is limited by uncertainty whether these mutations preceded the development of advanced liver disease. However, it does suggest that pre-S2 start codon mutations can be used as a predictor for development of advanced liver disease in HBeAg(+) patients (Figure 2E), but not in HBeAg(-) patients (Figure 2D). Interestingly, the frequency of T130, Q132, and A138 mutations was similar between the two groups (Table 5), indicating that the mutations in this region are not related to HBeAg seroconversion. In summary, our study demonstrated that the pre-S2 start codon mutation has high prevalence in Indonesia. This mutation may serve as biomarker for prediction of development of advanced liver disease in HBeAg(+) patients who are infected with HBV genotype B.

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COMMENTS

Background

Hepatitis B virus (HBV) infection is a serious worldwide health problem. Indonesia is one of the countries with high endemicity of hepatitis B related disease caused by HBV. Particular mutations of the HBV genome have been associated with severe liver disease. Studies on HBV molecular epidemiology associated with the development of progressive liver disease in Indonesia are still very scarce. This study is to characterize the prevalence of pre-S2 mutation in HBV associated with advanced liver disease in Indonesia.

Research frontiers

Pre-S mutations have been reported to be associated with advanced liver disease. Pre-S deletion and pre-S2 start codon mutations were the most common form of mutations at the pre-S region of HBV. Previous study had shown the low prevalence of pre-S deletion in Indonesian patients; however the prevalence of the pre-S2 start codon mutation has not been fully investigated. In this study, the authors demonstrated that the pre-S2 start codon mutation could serve as a biomarker for liver disease progression.

Innovations and breakthroughs

Previous studies have reported the high prevalence of HBV pre-S deletions and its association with advanced liver disease. But contrary to reports from studies of other populations, previous and current studies showed that pre-S2 start codon mutations and not pre-S deletion may serve as potential biomarkers for progressive liver disease in Indonesia.

Applications

By characterizing the prevalence of the HBV pre-S2 start codon mutation among different clinical diagnoses of different HBV genotype and hepatitis B e antigen (HBeAg) presence, the pre-S2 start codon mutation may serve as a potential biomarker for prediction of development of advanced liver disease in HBeAg(+) patients who are infected with HBV genotype B.

Terminology

HBV surface (HBs) gene has three different translation sites, pre-S1, pre-S2, and S, which produce large-, middle-, and small-HBs protein, respectively. Pre-S2 start codon mutation is a substitution or deletion of Methionine at the pre-S2 translation site which abolished the M HBs protein.

Peer review

The manuscript is interesting because the authors suggest a way to predict liver disease progression according to the appearance of a mutation at pre-S2 start codon in a subset of HBV infected patients.

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BRIEF ARTICLE

Oxaliplatin-induced severe anaphylactic reactions in metastatic colorectal cancer: Case series analysis

Jui-Ho Wang, Tai-Ming King, Min-Chi Chang, Chao-Wen Hsu

Jui-Ho Wang, Tai-Ming King, Min-Chi Chang, Chao-Wen Hsu, Division of Colorectal Surgery, Department of Surgery, Kaohsiung Veteran General Hospital, Kaohsiung 81346, Taiwan, China
Chao-Wen Hsu, Faculty of Medicine, School of Medicine, National Yang-Ming University, Taipei 112, Taiwan, China
Author contributions: Hsu CW wrote the manuscript and Wang JH revised the manuscript; King TM, Chang MC collected the data on the cases.
Correspondence to: Dr. Chao-Wen Hsu, Division of Colorectal Surgery, Department of Surgery, Kaohsiung Veteran General Hospital, Kaohsiung 81346, Taiwan, China. ss851124@gmail.com
Telephone: +886-7-3422121 Fax: +886-7-3422121
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Abstract

AIM: To investigate oxaliplatin-induced severe anaphylactic reactions (SAR) in metastatic colorectal cancer in a retrospective case series analysis and to conduct a systemic literature review.

METHODS: During a 6-year period from 2006 to 2011 at Kaohsiung Veterans General Hospital, a total of 412 patients exposed to oxaliplatin-related chemotherapy were retrospectively reviewed. Relevant English-language studies regarding life-threatening SAR following oxaliplatin were also reviewed in MEDLINE® and PubMed® search.

RESULTS: Eight patients (1.9%, 8 of 412 cases) were identified. Seven patients were successful resuscitated without any sequelae and one patient expired. We changed the chemotherapy regimen in five patients and rechallenged oxaliplatin use in patient 3. Twenty-three relevant English-language studies with 66 patients were reported. Patients received a median of 10 cycles of oxaliplatin (range, 2 to 29). Most common symptoms were respiratory distress (60%), fever (55%), and hypotension (54%). Three fatal events were reported (4.5%). Eleven patients (16%) of the 66 cases were rechallenged by oxaliplatin.

CONCLUSION: SAR must be considered in patients receiving oxaliplatin-related chemotherapy, especially in heavily pretreated patients. Further studies on the mechanism, predictors, preventive methods and management of oxaliplatin-related SAR are recommended.

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Key words: Oxaliplatin; Anaphylactic; Colorectal cancer; Metastasis

Peer reviewers: Susumu Ohwada, Associate Professor, Department of Surgery, Gunma University Graduate School of Medicine, 3-39-15 Shoma-Machi, Maebashi 371-8511, Japan; Cuong D Tran, PhD, Research Fellow, Affiliate Lecturer, University of Adelaide, Gastroenterology Unit, Children, Youth and Women's Health Service, 72 King William Rd, North Adelaide, SA 5006, Australia

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INTRODUCTION

Colorectal cancer (CRC) accounts for 10% to 15% of all cancers and is the third leading cause of cancer deaths in Taiwan. Oxaliplatin is a third generation platinum compound frequently used in the treatment of stage Ⅲ CRC as adjuvant chemotherapy[1] and stage Ⅳ advanced CRC[2]. Similar to other platinum compounds, oxaliplatin interacts with DNA to form intra-strand/inter-strand

DNA cross-linking that can affect DNA base pairing, replication, and gene transcription and cause cell death[3]. Among the common reasons for its withdrawal are frequent peripheral neuropathy, a delayed hypersensitivity reaction, and most troublesome, anaphylaxis when patients receive accumulated doses of oxaliplatin[4]. Hypersensitivity reaction and anaphylaxis refers to undesirable reactions produced by the normal immune system, including allergies and autoimmunity. These reactions may be damaging, uncomfortable, or occasionally fatal.

Multiple mechanisms of action have been proposed including the use of various neuroprotective agents in the hope of achieving adequate oxaliplatin doses with less neuropathy[5]. Much less is known about acute reactions such as anaphylaxis, and it is generally considered to be associated with immune-mediated effects[6,7]. The percentage of hypersensitivity reactions quoted in different studies ranges from 8% to 20%, but is usually around 10% to 12%[8]. Life-threatening severe anaphylactic reactions (SAR) have been reported but no systemic review of their incidence has been undertaken. Therefore, we performed a retrospective analysis of our patients who had been exposed to oxaliplatin and selected those who developed SAR requiring hospitalization with medical intervention. We also conducted a systemic literature review on this issue.

MATERIALS AND METHODS

Chart review

During a 6-year period from 2006 to 2011 at Kaohsiung Veterans General Hospital, a total of 412 patients exposed to oxaliplatin-related chemotherapy were retrospectively reviewed. Life-threatening SAR was defined as side effects including symptomatic bronchospasm, allergy-related edema/angioedema, hypotension or anaphylaxis (grade Ⅲ/Ⅳ anaphylactic reactions reference by NIH common Toxicity Criteria v3.0) requiring hospitalization and medical interventions[9]. The oxaliplatin-related chemotherapy regimen (FOLFOX) consisted of leucovorin 200 mg/m^2 as a 2-h infusion, and oxaliplatin 85 mg/m^2 given as a 2-h infusion in 500 mL of dextrose 5% *via* a Y-connector, followed by a 46-h infusion of 5-fluorouracil 2500 mg/m^2, repeated every 2 wk. Antiemetic prophylaxis with a 5HT3-receptor antagonist was administered. The use of implantable ports and disposable or electronic pumps allowed chemotherapy to be administered on an inpatient basis.

Data collection and literature review

Information collected included age, sex, allergy history, primary CRC site, tumor, nodes, metastasis classification, CRC stage, previous chemotherapy regimens, previous oxaliplatin-related chemotherapy cycles, oxaliplatin dosage, tumor response, onset time after oxaliplatin infusion, and outcome. Relevant English-language studies regarding life-threatening SAR following oxaliplatin were also reviewed. We searched the relevant studies by entering keywords "severe side effect after oxaliplatin", "life-threatening reaction after oxaliplatin" and "severe anaphylactic reaction after oxaliplatin" in MEDLINE® and PubMed® searches.

RESULTS

Patient characteristics

Eight patients (1.9%, 8 of 412 cases) were identified who developed life-threatening SAR, which occurred after infusion of oxaliplatin-related chemotherapy. The patients' characteristics were described in Table 1. There were 4 females and patients' age ranged from 36 to 72 years. Three patients had rectal cancer, 4 patients had sigmoid colon cancer, and 1 patient had descending colon cancer. Two patients had an allergy history to alcohol and flurbiprofen respectively. All patients had stage Ⅳ metastatic disease and received several lines of different chemotherapy regimens. Patients had received 5-29 cycles of oxaliplatin-related chemotherapy. Oxaliplatin dosages were 85 mg/m^2 in six patients and 90 mg/m^2 in one patient. Stable disease was achieved in three patients and progressive disease in five patients. Onset time after oxaliplatin infusion ranged from immediate to two hours. Seven patients were successfully resuscitated with oxygen support and medical interventions and fully recovered without any sequelae. However, one patient suffered from SAR and shock status 20 min after infusion of oxaliplatin. Despite cardiopulmonary resuscitation and use of inotropic agents, this patient expired 50 min later. We changed the chemotherapy regimen in five patients and rechallenged oxaliplatin use in patient 3. Because the patient 3's disease manifestations responded well to FOLFOX chemotherapy regimen, continuation was felt to be desirable. We have thus decided to attempt rechallenge of oxaliplatin by prolonging the infusion rate and using premedication with an additional 100 mg hydrocortisone plus diphenhydramine before the next treatment course. Fortunately, no anaphylactic reactions developed thereafter.

Literature review

Twenty-three relevant English-language studies, published from 1997-2011, regarding SAR following oxaliplatin-related chemotherapy were reported (Table 2). All studies were retrospective; few included the same patients. We found 59 reported cases that fitted the definition of life-threatening SAR from MEDLINE® and PubMed®[8-30]. Together with the 8 cases we presented, the median cycles of oxaliplatin given before SAR developed was 10 (range, 2-29). Most common symptoms were respiratory distress (60%), fever (55%), and hypotension (54%). Three fatal events were reported (4.5%). Eleven patients from these 66 cases were rechallenged with oxaliplatin.

DISCUSSION

According to previous studies, the estimated incidence of oxaliplatin-induced SAR was less than 2%[10,17,18,24,31]. In 2007, Lee *et al*[24] reported the incidence of SAR as 1.32%

Table 1 Clinical characteristics of patients with life-threatening severe anaphylactic reactions following oxaliplatin chemotherapy (n = 8)

Patient	Sex	Age (yr)	Allergy history	Primary CRC site	TNM classification	Stage	Previous chemotherapy regimens	Previous oxaliplatin chemotherapy cycles	Oxaliplatin dose (mg/m^2)	Tumor response	Presenting symptoms	Onset time	Outcome	Rechallenge
1	F	50	Alcohol	Rectum	T4N0M1	Ⅳ	FOLFIRI x12, FOLFOX x10	10	85	SD	Consciousness loss, dizziness, shock	30 min	Recovery	No
2	M	71	Nil	Rectum	T2N1M1	Ⅳ	FOLFOX x12, FOLFIRI + Bevacizumabx12, FOLFOX x1	13	85	PD	Consciousness loss, shock	20 min	Fatal	No
3	M	36	Nil	Sigmoid colon	T4N2M1	Ⅳ	FOLFIRI x13, FOLFOX + Bevacizumab x5, FOLFIRI + Cetuximab x5, FOLFOX x7	12	90	PD	Consciousness loss, respiratory distress, cold sweating	Immediate	Recovery	Yes
4	F	57	Nil	Sigmoid colon	T1N0M1	Ⅳ	FOLFIRI x5, FOLFOX x7, FOLFIRI + Bevacizumabx18, FOLFOX + Bevacizumab x1	8	85	PD	Respiratory distress, cold sweating	2 h	Recovery	No
5	F	68	Nil	Sigmoid colon	T3N2M1	Ⅳ	FOLFIRI x12, FOLFOX x8	8	85	SD	Angioedema, slurred speech, respiratory distress	30 m	Recovery	No
6	F	72	Nil	Descending colon	T4N1M1	Ⅳ	FOLFIRI x7, FOLFOX x3, FOLFIRI x12, FOLFOX x2	5	85	PD	Nausea, vomiting, shock,	10 min	Recovery	No
7	M	59	Flurbiprofen	Rectum	T3N2M1	Ⅳ	FOLFOX x19, FOLFIRI x8, FOLFOX x10	29	85	SD	Consciousness loss, respiratory distress, cold sweating	Immediate	Recovery	No
8	M	62	Nil	Sigmoid colon	T3N1M1	Ⅳ	FOLFIRI + Bevacizumabx6, FOLFOX x7	7	85	PD	Consciousness loss, respiratory distress, cold sweating	20 min	Recovery	No

M: Male; F: Female; CRC: Colorectal cancer; TNM: Tumor, nodes, metastasis; PD: Progressive disease; SD: Stable disease; FOLFIRI: Chemotherapy regimen including 5-fluorouracil, leucovorin, irinotecan; FOLFOX: Chemotherapy regimen including 5-fluorouracil, leucovorin, oxaliplatin.

Table 2 Studies on severe anaphylactic reactions following oxaliplatin, including data published from 1997 to 2012 in English (24 studies, *n* = 66)

Ref.	Published year/region	Patient No.	Age (yr)	Male/female	Previous oxaliplatin cycles	Oxaliplatin dose (mg/m^2)	Presenting symptoms	Onset time after oxaliplatin infusion	Outcome
de Gramont *et al*[10]	1997/France	5	NA	NA	NA	NA	NA	NA	Recovery
Tournigand *et al*[11]	1998/France	5	59-77	3/2	5-12	85-100	Reduced blood pressure, flushing, headache, tachycardia, respiratory distress	Immediate	Recovery
Larzillière *et al*[12]	1999/France	1	55	1/0	5	85	Flushing, profuse sweats, arterial hypertension, tachycardia	30 min	Recovery
Médioni *et al*[13]	1999/France	1	63	1/0	6	100	Visual disturbances, edema, tachycardia, severe hypotension, anaphylactic shock	Immediate	Recovery
Sørbye *et al*[14]	2000/Norway	1	40, 52	1/0	8	85	Severe thrombocytopenia	Immediate	Recovery
Santini *et al*[15]	2001/Italy	1	52	1/0	6	60	Chills, fever, nausea, vomiting, crampy abdominal pain, diarrhea, hypotension	15 min	Recovery
Schüll *et al*[16]	2001/Austria	1	54	1/0	5	85	Flush, generalised erythema of the trunk, nausea, hypotension	30 min	Recovery
Brandi *et al*[17]	2003/Italy	9	NA	NA	2-17	NA	Dyspnea, laryngospasm, agitation, tachycardia, precordial pain, erythema, sweating	NA	Recovery
Thomas *et al*[18]	2003/United States	1	50	0/1	9	NA	Fever, respiratory distress	2 h	Recovery
Lenz *et al*[19]	2003/Germany	2	NA	NA	NA	85	Severe abdominal, chest pain.	Immediate	Recovery
Bhargava *et al*[20]	2004/United States	1	50	0/1	12	NA	Palpitation, flushing, hypotensive, wheezing	15 min	Recovery
González-Mahave *et al*[21]	2005/Spain	2	43, 44	1/1	4, 11	NA	Respiratory collapse, fever	Immediate	Recovery
Maindrault-Goebel *et al*[22]	2005/France	3	NA	NA	NA	NA	Anaphylactic shock	NA	Recovery
Siu *et al*[8]	2006/Hong Kong	2	NA	NA	NA	100	Hypotension, oxygen desaturatio, full-blown anaphylactic reactions	NA	NA
Tze *et al*[23]	2006/China	1	60	0/1	12	NA	Anaphylactic shock	Immediate	Recovery
Lee *et al*[24]	2006/Taiwan	4	36-74	NA	6-7	85-100	Anaphylactic shock, hypertensive crisis	5-50 min	Recovery
Yanqi *et al*[25]	2007/China	1	52	1/0	6	150 mg	Anaphylactic shock	10 min	Recovery
Santodirocco *et al*[26]	2008/Italy	1	44	0/1	14	85	Acute thrombocytopenia, hemolysis, bleeding	1 h	Recovery
Shao *et al*[27]	2008/Taiwan	1	64	1/0	23	NA	Thrombocytopenia	1 h	Fatal
Chay *et al*[9]	2010/Singapore	11	36-75	4/7	NA	NA	Respiratory collapse, flushing, hypokalemia	NA	Recovery
Pietrantonio *et al*[28]	2010/Italy	1	NA	NA	NA	NA	Acute thrombocytopenia	NA	Recovery
Potenza *et al*[29]	2010/Italy	1	46	1/0	6	85	Respiratory collapse	10 h	Recovery
Teng *et al*[30]	2011/Taiwan	1	78	1/0	17	85	Pancytopenia, coagulopathy, intracranial hemorrhage	30 min	Fatal
Wang *et al*, this study	2012/Taiwan	7	36-72	3/4	5-29	85-90	Consciousness loss, chest tightness, cold sweating, nausea, vomiting, shock	immediately to 2 h	1 fatal

NA: Not applicable.

(4 of 303 cases) in Taiwan. In our study, the incidence is 1.9% (8 of 412 cases). Multiple suggestions to reduce the incidence of adverse reactions have been proposed, including the use of various neuroprotective agents, in the hope of achieving adequate oxaliplatin doses with less neuropathy[5]. Much less is known about acute reactions such as anaphylaxis, but this is generally considered to be associated with immune-mediated effects, as evidenced by detection of drug-dependent IgG antibodies with or without complement[6,7]. Two independent pathogenetic mechanisms have been proposed for this toxicity. Some authors described the formation of autoantibodies to erythrocytes and, more rarely, to platelets and neutrophils as a result of oxaliplatin adsorption on blood cells[32]. By contrast, other authors reported high levels of cytokines [i.e., interleukin (IL) 6, IL10 and tumor necrosis factor-α] suggesting that oxaliplatin-dependent toxicity may be triggered by a massive release of pro-inflammatory molecules[33].

In literature reviews, SAR developed after several cycles of oxaliplatin chemotherapy (median cycles before SAR is 10), suggesting a sensitization process of type I hypersensitivity due to the rapid appearance of symptoms[22,34]. Based on Chay *et al*[9], females appeared more prone to severe oxaliplatin reactions for which the reason remains unclear, and all females manifested acute hypokalemia. Recently reported ex-vivo work suggests that oxaliplatin may interfere with voltage-gated potassium channels[35] and hypothesizes that axonal membrane hyperpolarization[36,37] may account for the observed hypokalemia, with potassium ion channel activation resulting in an intracellular influx of potassium. However, in our study there were no such findings including female predominance and hypokalemia after the episode.

Theoretically, prolongation of the infusion rate with premedication including steroids and antihistamines could be a method to prevent SAR after oxaliplatin use. We adopted this strategy before rechallenging oxaliplatin in patient 3. However, in 2001 Stahl *et al*[38] reported that allergic reactions to oxaliplatin may still occur after steroid prophylaxis. In 2006, Siu *et al*[8] reported premedications with steroid and chlorpheniramine seemed ineffective in preventing SAR. In 2011, Siu *et al*[39] developed a simple rechallenge protocol for mild hypersensitivity reactions, including intravenous dexamethasone, diphenhydramine and ranitidine, as well as prolongation of the oxaliplatin infusion time with a high success rate of 70%. Why did the anaphylactic reactions disappear after rechallenge of oxaliplatin in patient 3? A possible explanation for the disappearance of symptoms may be the much lower peak plasma concentrations of the platinum compound and its metabolites in case of a protracted infusion[3], thus resulting in a minor and/or delayed, clinically negligible cytokine release reaction. In literature reviews, five of eleven rechallenged patients could tolerate oxaliplatin with no or minimal discomfort. However, there were still three patients developing SAR after receiving prolonged infusion of oxaliplatin. There were also reported cases initially having only a mild hypersensitivity reaction to oxaliplatin, who developed SAR after rechallenge with prolonged infusion schedule[22]. Therefore, it seems that prolonged infusion of oxaliplatin or using a desensitization program could only benefit a few patients who developed SAR. So, changing the chemotherapy regimen might be a better choice.

The mortality rate of oxaliplatin-related SAR was 4.5% (3 of 66 patients). In 2008, Shao *et al*[27] reported a fatal thrombocytopenia with a large intracranial hemorrhage with brain herniation after oxaliplatin chemotherapy. In 2011, Teng *et al*[30] reported another fatal pancytopenia with intracranial hemorrhage after oxaliplatin treatment. In our study, the patient who died initially presented with anaphylactic shock and loss of consciousness immediately after oxaliplatin infusion. All these three patients had been heavily pretreated with oxaliplatin and had received 23, 17 and 13 cycles of oxaliplatin treatment, respectively. To counteract the underlying immune-mediated mechanism, the use of steroids seems to be one of the most cost-effective approaches, especially when the patient's condition is life threatening[7,40]. This may also explain the fatalities in the patients reported by Shao *et al*[27], Teng *et al*[30] and our patient, who did not receive a steroid. Are there any predictors or risk factors for this rare but life-threatening event before oxaliplatin use? In 2011, Seki *et al*[41] reported a higher neutrophil count and lower monocyte count were two risk factors for grade 3/4 reactions in oxaliplatin-induced hypersensitivity reactions in Japanese patients. However, we didn't observe such a relationship in our study and the literature review.

Target therapy with monoclonal antibodies, including bevacizumab, cetuximab, and panitumumab, can also result in SAR[42]. Up till April 2012, there have been 14 698 people reported to have side effects when taking bevacizumab. Among them, 87 people (0.59%) have SAR[43]. In our study, one patient (patient 4) developed SAR after bevacizumab and oxaliplatin infusion. In our hospital, bevacizumab was started first and infused over 1-h. Oxaliplatin was infused after bevacizumab infusion. This patient developed SAR about 3 h after bevacizumab infusion and 2 h after oxaliplatin infusion. It is very difficult to differentiate the cause of SAR in this patient. But due to the time of onset of SAR, it is reasonable to suspect oxaliplatin.

Our study does have several limitations. First, being a retrospective review, it is difficult to confirm now whether those observed reactions are genuine hypersensitivity reactions or whether they developed as a result of oxaliplatin infusion only, although the temporal relationship between infusion and onset of reaction is suggestive. Therefore, it is possible that the risk may have been overestimated. We can also argue the other way round, that is, some mild reactions may have been missed resulting in underestimation.

In conclusion, SAR is rare but serious, and must be considered in patients receiving oxaliplatin-related che-

motherapy, especially in heavily pretreated patients. Physicians should be cautious when patients have repeated symptoms or signs of allergic reaction to oxaliplatin. At the moment, the mechanisms underlying oxaliplatin-related SAR remain uncertain. Prevention with prolongation of the infusion rate, steroid use and antihistamines are still in debate. Rechallenge with oxaliplatin is suggested only in carefully selected patients and should be used with caution. We recommend changing the chemotherapy regimen in patients experiencing oxaliplatin-induced SAR. Further extensive examinations with a large number of patients to determine the mechanism, the predictors, preventive methods and management strategy of oxaliplatin-induced SAR are recommended.

COMMENTS

Background

Oxaliplatin is a third generation platinum compound frequently used in the treatment of stage Ⅲ and stage Ⅳ colorectal cancer. Among the side effects of this agent, hypersensitivity reaction and anaphylaxis refers to undesirable reactions produced by the normal immune system, including allergies and autoimmunity. These reactions may be damaging, uncomfortable, or occasionally fatal. The percentage of hypersensitivity reactions quoted in different studies ranges from 8% to 20%. The authors presented their experience in this retrospective study and conducted a systemic review.

Research frontiers

Much less is known about acute reactions such as anaphylaxis, but it is generally considered to be associated with immune-mediated effects, as evidenced by detection of drug-dependent IgG antibodies with or without complement. Further extensive examination with a large number of patients to determine the mechanism, the predictors, preventive methods and management strategy of oxaliplatin-induced severe anaphylactic reactions (SAR) are recommended.

Innovations and breakthroughs

Life-threatening SAR have been reported but no systemic review had been performed. Here, the authors performed a retrospective analysis of the patients who had been exposed to oxaliplatin and selected those who developed SAR requiring hospitalization with medical intervention and conducted a systemic literature review on this issue.

Applications

Physicians should be cautious when patients have repeated symptoms or signs of allergic reaction to oxaliplatin. The effectiveness of prevention with prolongation of the infusion rate, steroid use and antihistamines is still in debate. Rechallenge of oxaliplatin is suggested only in highly selected patients and should be used with caution. The authors recommend changing the chemotherapy regimen in patients experiencing oxaliplatin-induced SAR.

Peer review

This manuscript is a retrospective analysis of oxaliplatin chemotherapy-induced SAR at Kaohsiung Veterans General Hospital in Taiwan. In addition, the authors have conducted a literature review on the same issue. This side effect is rare but is a life-threatening event; the authors have made some recommendations on the use of oxaliplatin as chemotherapy. This is important information which needs to be reported.

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BRIEF ARTICLE

Myofibrillogenesis regulator-1 overexpression is associated with poor prognosis of gastric cancer patients

Jing Guo, Bin Dong, Jia-Fu Ji, Ai-Wen Wu

Jing Guo, Jia-Fu Ji, Ai-Wen Wu, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Gastrointestinal Surgery, Peking University Cancer Hospital, Beijing Cancer Hospital and Institute, Beijing 100142, China
Bin Dong, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Pathology, Peking University Cancer Hospital, Beijing Cancer Hospital and Institute, Beijing 100142, China
Author contributions: Guo J, Ji JF and Wu AW designed the study; Dong B did pathological analysis; and Guo J performed the research, analyzed the data and wrote the manuscript.
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Correspondence to: Jia-Fu Ji, MD, PhD, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Gastrointestinal Surgery, Peking University Cancer Hospital, Beijing Cancer Hospital and Institute, Beijing 100142, China. jugularv@hotmail.com
Telephone: +86-10-88121122 Fax: +86-10-88122437
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Abstract

AIM: To investigate the expression of myofibrillogenesis regulator-1 (MR-1) in relation to clinicopathological parameters and postoperative survival in a group of Chinese patients with gastric cancer.

METHODS: In our previous study of human whole-genome gene expression profiling, the differentially expressed genes were detected in the gastric cancer and its adjacent noncancerous mucosa. We found that MR-1 was associated with the location and differentiation of tumors. In this study, MR-1 protein expression was determined by immunohistochemistry in specimens of primary cancer and the adjacent noncancerous tissues from gastric cancer patients. A set of real-time quantitative polymerase chain reaction assays based on the Universal ProbeLibrary-a collection of 165 presynthesized, fluorescence-labeled locked nucleic acid hydrolysis probes-was designed specifically to detect the expression of MR-1 mRNA. The correlation was analyzed between the expression of MR-1 and other tumor characteristics which may influence the prognosis of gastric cancer patients. A retrospective cohort study on the prognosis was carried out and clinical data were collected from medical records.

RESULTS: MR-1 mRNA and protein could be detected in gastric cancer tissues as well as in matched noncancerous tissues. MR-1 was up-regulated at both mRNA (5.459 ± 0.639 *vs* 1.233 ± 0.238, $P < 0.001$) and protein levels (34.2% *vs* 13.2%, $P = 0.003$) in gastric cancer tissues. Correlation analysis demonstrated that high expression of MR-1 in gastric cancer was significantly correlated with clinical stage ($P = 0.034$). Kaplan-Meier analysis showed that the postoperative survival of the MR-1 positive group tended to be poorer than that of the MR-1 negative group, and the difference was statistically significant ($P = 0.002$). Among all the patients with stage Ⅰ-Ⅳ carcinoma, the 5-year survival rates of MR-1 positive and negative groups were 50.40% and 12.70%, respectively, with respective median survival times of 64.27 mo (95%CI: 13.41-115.13) and 16.77 mo (95%CI: 8.80-24.74). Univariate and multivariate analyses were performed to compare the impact of MR-1 expression and other clinicopathological parameters on prognosis. In a univariate analysis on all 70 specimens, 6 factors were found to be significantly associated with the overall survival statistically: including MR-1 expression, depth of invasion, distant metastasis, lymph node metastasis, vascular invasion and the tumor node metastasis (TNM) stage based on the 7th edition of the International Union against Cancer TNM classification. To avoid the influence caused by univariate analysis, the expressions of MR-1 as well as other parameters were examined in multivariate Cox analysis. Clinicopathological variables that might affect the prognosis of gastric cancer patients were analyzed by Cox regression analysis, which showed that MR-1 expression

and TNM stage were independent predictors of postoperative survival. The best mathematical multivariate Cox regression model consisted of two factors: MR-1 expression and TNM stage. Our results indicated that MR-1 protein could act as an independent marker for patient overall survival [Hazard ratio (HR): 2.215, P = 0.043].

CONCLUSION: MR-1 is an important variable that can be used to evaluate the outcome, prognosis and targeted therapy of gastric cancer patients.

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Key words: Myofibrillogenesis regulator-1; Gastric cancer; Real-time quantitative reverse transcriptase-polymerase chain reaction; Immunohistochemistry; Poor prognosis

Peer reviewers: Islam Khan, Professor, Department of Biochemistry, Faculty of Medicine, Kuwait University, Jabrya Safat-13110, Kuwait; Dr. Guang-Wen Cao, MD, Professor, Department of Microbiology, Second Military Medical University, 800 Xiang Yin Road, Shanghai 200433, China

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INTRODUCTION

Gastric cancer remains the fourth most common malignancy, and the second leading cause of cancer-related death worldwide. It is estimated that one million new cases of gastric cancer occur each year[1,2], and most of them come from Asia (China, Japan and South Korea) and parts of Central and South America (Costa Rica, El Salvador and Columbia)[1]. More new cases of gastric cancer are diagnosed in China than in any other countries each year[3]. Many patients are found at an advanced stage with lymph node invasion and metastasis at their initial diagnosis. Despite a curative surgery and postoperative adjuvant therapy, nearly 60% of those patients succumb to the disease[4-6]. Cancer progression and metastasis is a highly complex multi-stage process. It involves increased cell adhesion, alterations in gene expression, and changes in cell motility. During invasion and metastasis, cancer cells move within tissues, and the invasion involves multiple processes regulated by various molecules[7]. As gastric cancer is featured as a heterogeneous disease in both histology and genetics, it is hard to predict patient outcome using the classic histological classifications. Gastric carcinogenesis is thought to be a multifactorial and multistep process involving the activation of oncogenes and the inactivation of tumor suppressor genes at different stages of gastric cancer progression. However, promising molecules that have clinicopathological/prognostic significance in gastric cancer are substantially limited. It is crucial to further understand the molecular mechanisms of cancer progression and the development involved in gastric cancer and to identify more valuable prognostic markers in order to improve patient prognosis as well as to provide novel promising therapy targets[8-11].

In our recent study, we utilized human whole-genome gene expression BeadChip of Illumina Company (Human 6-V2) to compare the differentially expressed genes between the adenocarcinoma of the esophagogastric junction (AEG) group and the distal gastric cancer group, and analyzed the difference of the genes related to gastric cancer and its adjacent noncancerous mucosa. There are 1121 differentially expressed genes from the BeadChip. By further analyzing the cDNA microarray data, we found that 15 genes were differentially expressed in AEG and distal gastric cancer, 90 genes were related to the differentiation of tumors. Myofibrillogenesis regulator-1 (MR-1) was associated with the location and differentiation of tumors simultaneously. In this study, we focused on the expression of MR-1 mRNA and protein in gastric cancers.

MR-1, which is mapped to 2q35, was first cloned from a human skeletal muscle cDNA library using polymerase chain reaction (PCR) and rapid amplification of cDNA ends (Genbank™ accession no. AF417001). MR-1 is composed of three distinct exons, in which exon 3 is unique when compared with other two genes, and encodes a protein of 142 amino acids with a hydrophobic transmembrane structure from 75 to 92 amino acids[12-15]. The transcription level of MR-1 in human tissues is especially high in myocardium and skeletal muscles as revealed by Northern blot and serial analysis of gene expression[12]. Overexpression of MR-1 could promote cancer cell proliferation and migration in human hepatoma G2 (HepG2) cells[16]. MR-1 might promote cancer cell proliferation by binding to specific proteins, such as eukaryotic initiation factor 3 that is highly correlated with tumor cell growth and invasion regulation[17]. Also, overexpression of MR-1 can activate the nuclear factor κB signaling pathway, which is correlated with a wide variety of diseases, including cancer, inflammation, and autoimmune diseases[18].

Taking all the evidences listed above into account, we hypothesized that MR-1 may take part in the development and progression of gastric cancer. On the basis of these studies, we used real-time quantitative reverse transcriptase-polymerase chain reaction (qPCR) and immunohistochemistry to examine the expression of MR-1 in gastric cancer samples and adjacent normal tissues. Our study was the first attempt to investigate the relationship between MR-1 expression and prognosis of gastric cancer patients with complete clinical and follow-up data. We analyzed MR-1 protein expression and studied the relationship between MR-1 expression and survival. We also evaluated the possible associations between MR-1 protein expression and clinicopathological characteristics.

MATERIALS AND METHODS

Patients and tissue specimens

In this retrospective study, a consecutive series of 70 paired tissue specimens were collected from the patients with gastric cancer who received subtotal or total gastrec-

tomy at the Peking University Cancer Hospital in Beijing between January 2004 and December 2005. Written informed consent was obtained before sample collection and this study was approved by the Ethics Committee of Peking University. There were 45 males and 25 females with a mean age of 56 years (range: 26-81 years). None of the patients had undergone either chemotherapy or radiotherapy before surgery and there was no other co-occurrence of diagnosed cancers. A number of clinicopathological variables such as gender, age, tumor location, histological type, tumor-node-metastasis (TNM) stage, depth of tumor invasion, lymph node metastasis, distant metastasis and vascular invasion were obtained from the histopathological records and included for survival analysis.

We classified the postoperative staging of gastric cancer according to 7th American Joint Committee on Cancer (AJCC) TNM staging classification for carcinoma of the stomach[19]. There were 6 patients with stage Ⅰ, 15 patients with stage Ⅱ, 43 patients with stage Ⅲ, and 6 patients with stage Ⅳ carcinoma. After gastrectomy, resected specimens were processed routinely for histopathological assessment; necrotic hemorrhage and connective tissues were removed and each paired bulk sample [tumor samples (T)/matched normal samples (N)] was immediately put into liquid nitrogen and stored at -80 ℃ until processed. The resected specimens of gastric cancer were also routinely subjected to macroscopic pathological assessment and fixed with 10% formalin in phosphate buffered saline (PBS) for immunohistochemistry. All tissue specimens were formalin-fixed and paraffin-embedded. Formalin fixed tissue sections were stained with haematoxylin and eosin and classified by a pathologist. These results were compared with the histopathologial records from Peking University Cancer Hospital. Final pathology was determined by consensus and reviewed if necessary. The patients were followed up from a period of 2.23 to 89.07 mo (mean, 30.78 mo). Follow-up was managed through correspondence, over the telephone or in the clinic every 3 to 6 mo for 5 years and half a year thereafter. In the clinic, history enquiry, physical examination, complete blood count, biochemical tests, imaging studies and endoscopy were routinely completed. All gastric cancer patients in our study were followed up regularly and follow-up information was complete. The primary endpoint of the follow-up was death of gastric cancer patients. Patients who did not die as a result of gastric cancer were excluded.

Isolation of total RNA

Total RNA was extracted according to the manufacturer's instructions (TRIzol, Invitrogen, United States). The integrity of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands were visualized under ultraviolet (UV) light. The RNA was stored at -80 ℃ in RNase-free water until reverse transcription or fluorescence labeling.

Semiquantitative reverse transcription-polymerase chain reaction

Reverse transcription (RT) was performed in a 25 μL reaction volume with 2 μg total RNA treated with 0.5 μg of Oligo (dt), 200 U Moloney murine leukemia virus reverse transcriptase, 25 U RNase inhibitor and 2.5 mmol dNTP to synthesize the first-strand cDNA (Promega, United States), according to the manufacturer's recommendations. The reaction system was incubated at 70 ℃ for 5 min (primer annealing), 42 ℃ for 1 h (synthesis) and resulting cDNA was stored at -20 ℃. The resulting cDNA was subjected to PCR for the evaluation of the relative expression levels of β-actin (as an internal control) and MR-1. PCR was done using 1 unit HotMaster Taq DNA polymerase (Qiagen, Germany) and 1:20 of the reverse transcription reaction, with an initial hot start of 5 min at 95 ℃ followed by 30 s denaturation. Primers, annealing and extension temperatures, and number of cycles used (chosen for the exponential phase of amplification) were as follows: (1) β-actin forward primer: CATGCCATCCTGCGTCTGGAC, reverse primer: CACGGAGTACTTGCGCTCAGGAGG; 55 ℃, 45 s, 72 ℃, 45 s, 28 cycles, 72 ℃, 5-min bands of 275 bp; and (2) MR-1 forward primer: GCTTTGCAGGTGTGGTGGAG, reverse primer: AGGAACGGGTTGTAGGAGCG; 52 ℃, 40 s, 72 ℃, 45 s, 35 cycles, 72 ℃, 5-min bands of 133 bp. Two bands were detected at 133 bp and 275 bp corresponding to the molecular weight marker. PCR products were electrophoresed on 1.5% agarose gels with 0.01% ethidium bromide. Band intensities were measured under UV light using Gel Analyst software (UVP, Upland, United States).

Real-time quantitative reverse transcription-polymerase chain reaction

Real-time qPCR was performed in an ABI Prism 7500 HT (Applied Biosystems, Foster City, CA) with a Universal ProbeLibrary (UPL) probe (Roche). Primers and probe were designed by online Roche Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000). The primers for real-time qPCR were: MR-1 forward: 5'-CTTCTCAGGGGACCTGCTCT-3', reverse: 5'-TCAGCATGGTCTCTGCATTG-3'; β-actin forward: 5'-CCAACCGCGAGAAGATGA-3', reverse: 5'-CCAGAGGCGTACAGGGATAG-3'. UPL probe #76 was designed for MR-1 and UPL probe #64 for β-actin. All other reaction conditions were as described by the manufacturer. The cDNA was denatured and the Taq DNA polymerase was activated for 10 min at 95 ℃, and the cycling conditions were set as follows: 40 cycles of denaturation at 95 ℃ for 30 s, annealing at 52 ℃ for 30 s, extension at 72 ℃ for 30 s and a final step at 42 ℃ for 2 min. Standard curves were determined by running a dilution series on the housekeeping gene (β-actin) and target gene. The experiments were repeated three times independently.

Immunohistochemistry

Immunohistochemical analysis was done to study the altered protein expression in the 70 specimens of human gastric cancer tissues and non-cancerous gastric tissue controls. Four μm sections from formalin-fixed and paraffin-embedded tissues were mounted on poly-L-lysine-coated slides, baked overnight at 50 ℃ and then deparaffinized in xylene and rehydrated through alcohol to distilled wa-

ter. After hydration, endogenous peroxidase activity was blocked by incubation with 3% (v/v) hydrogen peroxidase (H_2O_2) for 20 min at room temperature. Standard antigen retrieval was then performed with heat-induced epitope retrieval (HIER) by heating the slides immersed in retrieval solution (pH 6.0) in a pressure boiler. After boiling, the slides remained in the pressure boiler for 3 min and then gradually cooled at room temperature. For the detection of MR-1, after washing with PBS three times, the sections were incubated with the polyclonal goat anti-human MR-1 antibody (HPA017068-100UL, Sigma, Germany) at 4 ℃ overnight. Then, the slides were incubated with peroxidase-labeled polymer conjugated to poly peroxidase-anti-mouse/rabbit IgG (PV-9000, Zhongshan Biotechnology Company, Beijing, China) at 37 ℃ for 30 min followed by a gentle rinse with washing buffer three times. 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining reaction was then performed and followed by Meyer hematoxylin counterstaining. The slides were then dehydrated, cleared and mounted as usual. For negative controls, the primary antibody was replaced by non-immune rabbit serum to confirm the specificity. Internal positive control was used for quality assurance. MR-1 staining was principally evaluated according to the scoring criteria. The information recorded was: subcellular location (nuclear and/or cytoplasmic), intensity of staining (negative, weak, moderate or strong) and percentage of positive immunoreactive cells. The positive group referred to the cases with > 10% cells having positive immunoreactivity. The rest was defined as negative. The slide evaluation was performed by two pathologists, and both pathologists gave almost identical reports with only minor differences. A consensus regarding controversial cases was reached after discussion.

Statistical analysis

All statistical analyses were performed using SPSS statistical analysis software, version 16.0 (SPSS, Chicago, IL, United States). A paired-samples *t* test was used to compare the MR-1 mRNA levels in the tumor tissue samples and their paired adjacent non-tumor tissue samples. Regarding MR-1 expression and the clinicopathological variables, data were cross-tabulated and a χ^2 test was performed. Cumulative survival was estimated by the Kaplan-Meier method and comparisons between groups were made with a log-rank test. Postoperative survival was measured from the date of first surgery to the date of death of gastric cancer, or the last date of information collection if no end event was documented. A multivariate analysis of Cox proportional hazards regression model (backward, stepwise) was performed to assess the influence of each variable on survival. $P < 0.05$ was considered statistically significant.

RESULTS

Myofibrillogenesis regulator-1 mRNA levels are increased in gastric cancers

We randomly selected 60 specimens from the total samples, including 30 gastric cancer tissues and 30 matched noncancerous tissues to investigate the *MR-1* mRNA expression level using semiquantitative RT-PCR. As shown in Figure 1A, *MR-1* mRNA could be detected in both gastric cancer tissues and matched noncancerous tissues. However, a significant increase in the levels of *MR-1* mRNA expression was observed in primary gastric cancer compared with matched normal tissues.

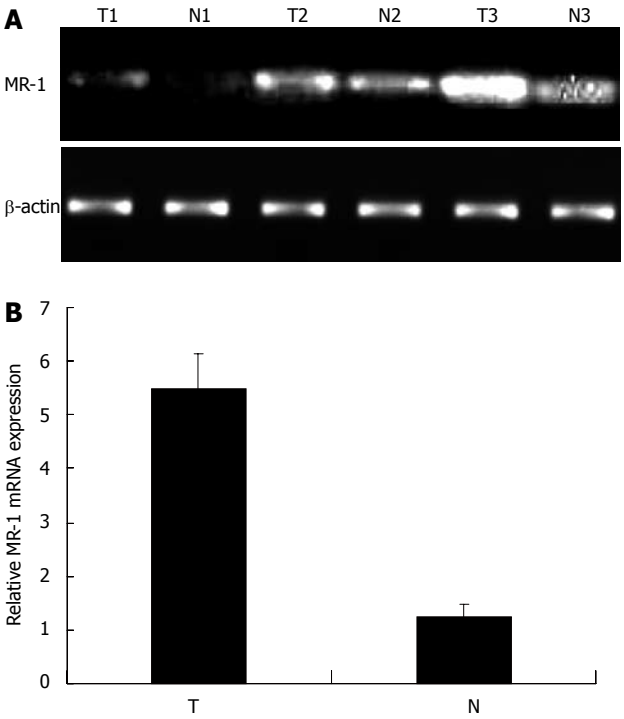


Figure 1 Expression of myofibrillogenesis regulator-1 mRNA in adjacent noncancerous mucosa and gastric cancers. A: Expression pattern of myofibrillogenesis regulator-1 (MR-1) in gastric cancer specimens by semiquantitative reverse transcription-polymerase chain reaction (PCR) (representative PCR results from 3 patients). The expression level of MR-1 mRNA showed significant difference between gastric cancer tissues and corresponding noncancerous gastric tissues; B: Results of real-time quantitative PCR assay. MR-1 mRNA expression in tumor tissue was frequently higher than that in matched normal mucosa ($P < 0.001$; Student's *t* test). β-actin was used as a control. N: Noncancerous gastric tissues; T: Gastric cancer tissues.

To validate the results of semiquantitative RT-PCR, we examined the *MR-1* mRNA expression level with real-time qPCR in the 30 paired clinical samples chosen randomly from the total cases. By real-time qPCR analysis, we found that the level of *MR-1* mRNA was increased remarkably in gastric cancer tissues. Expression of *MR-1* mRNA was measured in triplicate, and then normalized relative to the reference gene β-actin (divided by the expression level of human β-actin). The average ratios of *MR-1* mRNA to *β-actin* mRNA in gastric cancer tissues and noncancerous gastric tissues were 5.459 ± 0.639 and 1.233 ± 0.238, respectively (Figure 1B), which suggested that the expression level of *MR-1* mRNA was significantly higher in gastric cancer tissues than in the corresponding noncancerous gastric tissues ($P < 0.001$).

Myofibrillogenesis regulator-1 protein expression pattern in primary gastric cancer

MR-1 protein expression was detected in the nuclei and cytoplasm of both adjacent noncancerous mucosa and gastric cancer cells. However, the positive rate of MR-1

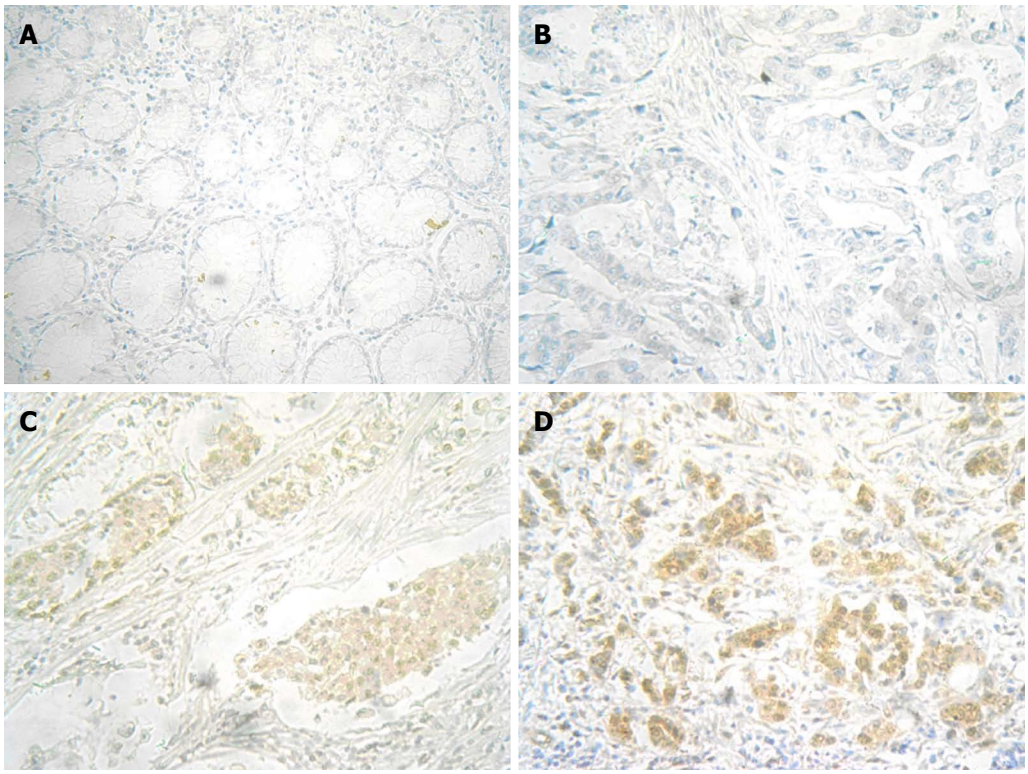


Figure 2 Myofibrillogenesis regulator-1 protein expression determined by immunohistochemical staining in adjacent noncancerous mucosa and gastric cancers. A: Negative expression in adjacent noncancerous mucosa (× 200). No myofibrillogenesis regulator-1 (MR-1) expression was detected; B: Negative expression in gastric cancers (× 200). No MR-1 expression was detected; C: Weak positive expression in gastric cancer (× 200); D: Moderate positive expression in gastric cancer (× 200). MR-1 protein was expressed in cytoplasm of the cells in (C) and (D).

expression in gastric cancer cells was much higher than that in adjacent noncancerous mucosa (34.2% *vs* 13.2%, $P = 0.003$, Figure 2).

Association of myofibrillogenesis regulator-1 protein expression with clinicopathological variables and postoperative survival in gastric cancer

We investigated the association of MR-1 protein expression with clinicopathological variables and postoperative survival. Correlation analysis demonstrated that high expression of MR-1 in gastric cancer was significantly correlated with TNM stage ($P = 0.034$). It suggested that MR-1 protein expression had no obvious association with other clinicopathological variables except TNM stage (Table 1).

Kaplan-Meier analysis showed that the postoperative survival of the MR-1 positive group tended to be poorer than that of the MR-1 negative group, and the difference was statistically significant ($P = 0.002$). For all the patients with stages I -IV carcinoma, the 5-year survival rates of MR-1 positive and negative groups were 50.40% and 12.70%, respectively, with respective median survival times of 64.27 mo (95%CI: 13.41-115.13) and 16.77 mo (95%CI: 8.80-24.74, Figure 3).

We performed univariate and multivariate analyses to compare the impact of MR-1 expression and other clinicopathological parameters on prognosis. In the univariate analysis on all 70 specimens, 6 factors were found to have statistically significant associations with overall survival: MR-1 expression, depth of invasion, distant metastasis, lymph node metastasis, vascular invasion and the TNM stage based on the 7th edition of the International Union Against Cancer TNM classification (Table 2). To avoid the influence caused by univariate analysis, the expressions of MR-1 as well as other parameters were examined in multivariate Cox analysis. Clinicopathological variables that might affect the prognosis of gastric cancer patients were analyzed by Cox regression analysis, which showed that MR-1 expression and TNM stage were independent predictors of postoperative survival (Table 2). Therefore, the best mathematical multivariate Cox regression model consisted of two factors: MR-1 expression and TNM stage. In the testing set, MR-1 was again found to be a significant independent prognostic factor for poor prognosis (hazard ratio, 2.125; 95%CI: 1.023-4.410, $P = 0.043$; Table 2) in Chinese gastric cancer patients.

DISCUSSION

Gastric cancer is reported to be the second most common cause of cancer-related death worldwide. For most cases, at the advanced stage when diagnosed, surgery is the only curative procedure for localized gastric cancer. A high recurrence rate, a low survival rate, and a poor prognosis were found in the advanced gastric cancer patients because currently available agents are not very effective. Thus, treatment of gastric tumors remains a challenge for physicians. To identify more effective approaches for cancer treatment, new targeted therapies for advanced gas-

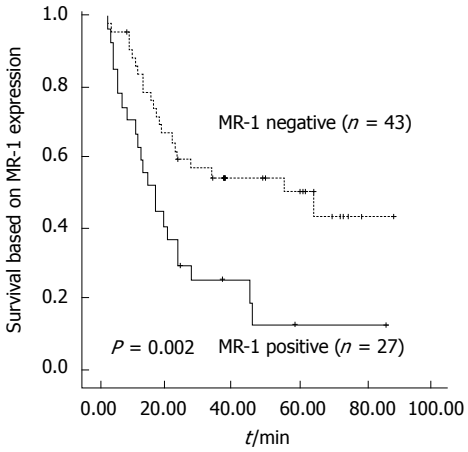


Figure 3 Postoperative survival curves in patients with stage Ⅰ-Ⅳ carcinoma. Myofibrillogenesis regulator-1 (MR-1) protein positive expression refers to cases showing weak and moderate staining.

tric cancers are needed. Right now, targeted therapy for advanced gastric carcinoma relies on target gene status evaluation[20,21]. Because of the cellular migration and metastasis, the mortality rate of gastric cancer remains high. The development of gastric cancer is a comprehensive action associated with multiple factors, such as inhibition of tumor suppressor genes, overexpression of related genes, and a failure to regulate cell proliferation. Therefore, it is urgently needed to find a sensitive biomarker for the detection of gastric cancer at the curative stage.

Li *et al*[12] identified a novel human gene, MR-1, from a human skeletal muscle cDNA library. MR-1 is located on human chromosome 2q35, having three alternatively spliced forms[22]. Based on our recent findings that MR-1 was associated with the location and differentiation of tumors simultaneously, and that MR-1 played a role in promoting the proliferation and invasion in some types of human cancers[14,18], we designed this study to comprehensively investigate the association between MR-1 and gastric cancer and the roles it may play in the pathogenesis or disease progression.

Real-time qPCR procedure combined with the Universal ProbeLibrary (UPL) technology has been successfully employed for both detection and quantification of expression levels of numerous genes, and mammalian and human viral pathogens[23-27]. UPL is a collection of 165 presynthesized, fluorescence-labeled DNA/locked nucleic acid (LNA) hybrid hydrolysis probes, which have been selected carefully to detect 8- and 9-mer motifs that are very prevalent in the transcriptomes, ensuring optimal coverage of all transcripts in a given transcriptome. On the basis of the MR-1 and β-actin sequences, UPL probe #76 and #64 with each specific PCR primer pairs were obtained from universal probe library database (Roche Diagnostics). In the present study, the prognostic relevance of MR-1 expression in gastric cancer was reported for the first time. It has been shown that MR-1 mRNA was overexpressed in gastric tumors compared with adjacent normal gastric tissues from the same individuals, in agreement with the data obtained from our microarray analysis.

In this study, immunohistochemistry was used to analyze the levels of MR-1 expression in 70 clinicopathologically characterized gastric cancer cases. The positive rate of MR-1 protein expression in gastric cancer cells was found much higher than in adjacent noncancerous mucosa. The results indicate that there are certain functions of MR-1 protein that are highly expressed in gastric cancer cells, which was confirmed by postoperative survival analysis in our study. To the best of our knowledge, there has been no report examining the role of MR-1 in gastric cancer. This is the first study showing that expression of MR-1 is increased in gastric cancer tissues compared with benign control tissues.

Table 1 Association of myofibrillogenesis regulator-1 protein expression with clinicopathological variables of gastric cancer patients

Variables	Cases	MR-1 negative *n* = 43 (%)	MR-1 positive *n* = 27 (%)	*P* value
Gender				0.855
Male	45	28 (62.22)	17 (37.78)	
Female	25	15 (60.00)	10 (40.00)	
Location				0.246
Upper	8	3 (37.50)	5 (62.50)	
Lower	62	40 (64.52)	22 (35.48)	
Age (yr)				0.400
< 55	25	17 (68.00)	8 (32.00)	
⩾ 55	45	26 (57.78)	19 (42.22)	
TNM stage				0.034
Ⅰ + Ⅱ	21	17 (80.95)	4 (19.05)	
Ⅲ + Ⅳ	49	26 (53.06)	23 (46.94)	
Depth of invasion				0.809
T1 + T2	12	7 (58.33)	5 (41.67)	
T3 + T4	58	36 (62.07)	22 (37.93)	
Lymph node metastasis				0.347
Negative	12	9 (75.00)	3 (25.00)	
Positive	58	34 (58.62)	24 (41.38)	
Distant metastasis				0.196
M0	64	41 (64.06)	23 (35.94)	
M1	6	2 (33.33)	4 (66.67)	
Vascular invasion				0.887
Negative	41	25 (60.98)	16 (39.02)	
Positive	27	16 (59.26)	11 (40.74)	
Differentiation				0.278
Well and moderately	3	1 (33.33)	2 (66.67)	
Poorly	60	36 (60.00)	24 (40.00)	
Others	5	4 (80.00)	1 (20.00)	

TNM: Tumor node metastasis; MR-1: Myofibrillogenesis regulator-1.

The clinical significance of MR-1 protein expression was studied. The result reveals that high expression of MR-1 in gastric cancer is significantly correlated with TNM stage (P = 0.034), but has no obvious association with other clinicopathological variables related to the prognosis of gastric cancer patients, such as depth of invasion and vascular invasion[28,29].

AEG has a different clinicopathological feature and poor prognosis compared with the distal gastric cancer. In this study, 5 (62.50%) of 8 cases of AEG highly expressed MR-1 protein, whereas weak expression was detected only in 22 (35.48%) of 62 cases of the distal gastric cancer. Expression was detected in two (66.67%) of three cases of well and moderately differentiated gastric cancers, whereas 24 (40.00%) of 60 cases of the poorly differentiated gastric cancer expressed MR-1 protein. These findings were in accordance with the data obtained from our microarray

Table 2 Multivariate survival analysis of the prognosis factors by Cox regression analysis

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
MR-1 expression (positive *vs* negative)	2.502 (1.365-4.586)	0.003	2.125 (1.023-4.410)	0.043
TNM stage (stages Ⅲ-Ⅳ *vs* Ⅰ-Ⅱ)	12.608 (3.845-41.345)	< 0.001	5.214 (1.155-23.526)	0.032
Depth of invasion (T3 + T4 *vs* T1 + T2)	7.094 (1.709-29.441)	0.007	3.340 (0.612-18.212)	0.164
Distant metastasis (M1 *vs* M0)	0.395 (0.166-0.943)	0.036	0.579 (0.199-1.686)	0.316
Gender (male *vs* female)	1.180 (0.635-2.192)	0.601	0.783 (0.376-1.631)	0.514
Age (yr) (< 55 *vs* ≥ 55)	0.603 (0.324-1.122)	0.110	0.717 (0.340-1.514)	0.383
Location (upper *vs* lower)	1.740 (0.726-4.168)	0.214	1.136 (0.449-2.876)	0.787
Vascular invasion (positive *vs* negative)	0.517 (0.267-1.001)	0.046	0.964 (0.456-2.038)	0.924
Lymph node metastasis (positive *vs* negative)	4.973 (1.529-16.176)	0.008	1.158 (0.249-5.382)	0.852
Differentiation (well differentiated *vs* poorly differentiated)	0.774 (0.478-1.254)	0.298	0.831 (0.485-1.426)	0.502

HR: Hazard ratio; CI: Confidence interval; TNM: Tumor node metastasis; MR-1: Myofibrillogenesis regulator-1.

analysis. However, data derived from these studies were statistically not significant, which may be because of the small sample size. Therefore, the accuracy of this first explorative result should be further tested in a larger validation study.

With respect to the results of survival analysis, in patients with stages Ⅰ-Ⅳ gastric cancer, the 5-year survival rate for those with high MR-1 expression was significantly lower than that of patients with low MR-1 expression. The results showed that MR-1 was up-regulated in gastric cancer tissues compared with normal gastric tissues and correlated significantly with prognosis. Multivariate analysis suggested that MR-1 expression and TNM stage were independent prognostic indicators for gastric cancer. The relative risk of death in patients with MR-1-positive tumors was 2.125 times higher than that in the patients with MR-1-negative tumors [hazard ratio (HR) = 2.125, 95%CI: 1.023-4.410]. Depth of invasion, distant metastasis, lymph node metastasis and vascular invasion, which were significant prognostic factors in the univariate analysis, showed no significant influence on survival in the multivariate analysis, possibly because their prognostic value was overlapped by the TNM stage. Thus, MR-1 is a potentially novel therapeutic target for the treatment of gastric cancer.

The mechanism by which MR-1 promotes tumorigenesis and cancer progression has not been well elucidated. The interaction of MR-1 with sarcomeric structural proteins involved in muscle contraction and its presence in human myocardial myofibrils indicate that MR-1 could regulate contractile proteins in the myocardium and might be associated with cardiac hypertrophy. Myosin light chain-2 (MLC-2) plays an important role in cell migration from solid cancers such as ovarian tumor, and its dephosphorylation could induce apoptosis[30]. A study showed that MLC-2 may regulate cell proliferation and migration by interacting with MR-1[31]. Knockdown of MR-1 expression in human hepatoma HepG2 cells inhibits cell migration and proliferation both *in vitro* and *in vivo*. The mechanism underlying this action is that MR-1 induces MLC-2 activation, subsequently stimulates stress fiber formation, and indirectly activates the focal adhesion kinase/protein kinase B (FAK/Akt) signaling pathway to promote cell migration and proliferation[15]. Further studies are needed to define the molecular mechanisms that govern the potential role of MR-1 expression in gastric cancer progression, clarify whether MR-1 is an early diagnostic marker for gastric cancer and to assess its full therapeutic potential.

In conclusion, our data show that a subset of patients with gastric cancer have MR-1 overexpression, which is associated with an aggressive clinical course and poor overall survival. Thus, MR-1 may be a novel biological marker and potential therapeutic target for the treatment of gastric cancer. It could also be used to monitor the effect of anti-cancer therapies. The results of our study suggest that overexpression of MR-1 in gastric cancer tissues might play an important role in the progression and metastases of the disease and that MR-1 may be a useful prognostic and survival indicator. These findings may help us explore novel therapeutic modalities and prognostic predictors for gastric cancer patients, thus improving the treatment outcomes. This is the first report to suggest a relationship between MR-1 and prognosis of patients with gastric cancer, and further prospective investigations would be worth doing in clinical settings.

COMMENTS

Background

Gastric cancer is one of the commonest malignant tumors in the alimentary tract and is characterized by delayed clinical presentation, rapid progression, and poor survival. Although this neoplasm is a serious public health problem due to its high incidence and mortality, little is known about the molecular events involved in gastric carcinogenesis.

Research frontiers

Gastric cancer, similar to other neoplasms, is a multifactorial disease that results from a combination of environmental factors and the accumulation of generalized and specific genetic and epigenetic alterations. Myofibrillogenesis regulator-1 (MR-1) protein family has been recently found associated with carcinogenesis, but not in the stomach. In this study, the authors evaluated mRNA and protein expression of MR-1 in gastric neoplasms and corresponding non-neoplastic samples.

Innovations and breakthroughs

No previous study has evaluated the gene and protein expression of MR-1 in gastric carcinogenesis. The findings from this study suggested that MR-1 may play an important role in the development of gastric cancer and it is a potential indicator that could be used to evaluate the outcome of gastric cancer treatment.

Applications

These results suggest that overexpression of MR-1 is associated with clinical stage and serves as a prognostic factor in patients with gastric cancer.

Terminology

MR-1, which is mapped to 2q35, was first cloned from a human skeletal muscle cDNA library using PCR and rapid amplification of cDNA ends (Genbank™ accession no. AF417001). It encodes a protein of 142 amino acids with a hydrophobic transmembrane structure from 75 to 92 amino acids.

Peer review

The study discovered some differential expression genes through bioinformatics and elucidated the expression level of MR-1 in gastric cancer and evaluated the link between MR-1 and the poor outcome of gastric cancer. The major finding of this study was that MR-1, which up-regulated in gastric cancer tissues comparing with matched non-tumor tissues, might be a novel prognostic marker for gastric cancer.

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BRIEF ARTICLE

Identification of deregulated miRNAs and their targets in hepatitis B virus-associated hepatocellular carcinoma

Wen Wang, Lan Juan Zhao, Ye-Xiong Tan, Hao Ren, Zhong-Tian Qi

Wen Wang, Lan-Juan Zhao, Hao Ren, Zhong-Tian Qi, Department of Microbiology, Shanghai Key Laboratory of Medical Biodefense, Second Military Medical University, Shanghai 200433, China
Ye-Xiong Tan, Laboratory of Signal Transduction, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200433, China
Author contributions: Wang W and Zhao LJ contributed equally to the work, Wang W performed the research, drafted the article; Zhao LJ performed the research and revised the article; Tan YX performed the research; Ren H and Qi ZT designed the research; and all the authors have read and approved the final version to be published.
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Correspondence to: Zhong-Tian Qi, MD, PhD, Department of Microbiology, Shanghai Key Laboratory of Medical Biodefense, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, China. qizt@smmu.edu.cn
Telephone: +86-21-81870988 Fax: +86-21-81870988
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Abstract

AIM: To identify the differentially expressed miRNAs and their targets in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC).

METHODS: Six hundred and sixty seven human miRNAs were quantitatively analyzed by Taqman low-density miRNA array (TLDA) in HBV-HCC tissues. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were used to analyze the significant function and pathway of the differentially expressed miRNAs in HBV-HCC. TargetScan software was used to predict the targets of deregulated miRNAs. Western blotting and luciferase assay were performed to verify the targets of these miRNAs.

RESULTS: Ten up-regulated miRNAs (miR-217, miR-518b, miR-517c, miR-520g, miR-519a, miR-522, miR-518e, miR-525-3p, miR-512-3p, and miR-518a-3p) and 11 down-regulated miRNAs (miR-138, miR-214, miR-214#, miR-199a-5p, miR-433, miR-511, miR-592, miR-483-3p, miR-483-5p, miR-708 and miR-1275) were identified by Taqman miRNAs array and confirmed quantitatively by reverse transcription polymerase chain reaction in HCC and adjacent non-tumor tissues. GO and KEGG pathway analysis revealed that "regulation of actin cytoskeleton" and "pathway in cancer" are most likely to play critical roles in HCC tumorigenesis. MiR-519a and ribosomal protein S6 kinase polypeptide 3 (RPS6KA3) were predicted as the most significant candidates by miRNA-mRNA network. In addition, cyclin D3 (CCND3) and clathrin heavy chain (CHC), usually up-regulated in HCC tissues, were validated as the direct target of miR-138 and miR-199a-5p, respectively.

CONCLUSION: Our data suggest an importance of miR-138 and miR-199a-5p as well as their targets CCND3 and CHC in HCC tumorigenesis, and may provide more evidence for reliability of integrative bioinformatics analysis.

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Key words: Hepatocellular carcinoma; miR-138; miR-199a-5p; Cyclin D3; Clathrin heavy chain; Bioinformatics; Taqman array

Peer reviewer: Ferenc Sipos, MD, PhD, Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi u. 46, 1088 Budapest, Hungary

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide[1]. The development and progression of HCC is characterized by a multi-stage process, which is believed to involve the deregulation of genes that are critical to cellular processes such as cell cycle control, cell growth, apoptosis, and cell migration and spreading. In the past decades, studies have focused on investigating the effect of genes and proteins on the development and progression of HCC[2]. Recently, an increasing number of reports have described microRNAs (miRNAs) that are implicated in HCC progression[3].

MiRNAs are endogenous non-coding RNAs (20-22 nucleotides) which regulate gene expression by catalyzing the cleavage of messenger RNA (mRNA) or repressing mRNA translation[4]. Increasing evidence showed that miRNAs play significant roles in cell development, differentiation and communication[5]. Deregulation of miRNAs has also been observed in a wide range of human diseases, including cancer[6]. In human cancer, miRNAs can function as oncogenes or tumor suppressor genes during tumor development and progression[7].

In this study, the expression of 667 miRNAs was profiled in human HCC and adjacent non-tumor tissues. A set of significantly differentially expressed miRNAs was identified in HCC tissues. Furthermore, a global analysis of miRNA-regulated signaling pathways and related genes was performed on the basis of miRNA expression profiles and bioinformatics interpretation. Cyclin D3 (CCND3) and clathrin heavy chain (CHC) which had been previously described in HCC tumorigenesis were further validated to be the direct target of miR-138 and miR-199a-5p, respectively.

MATERIALS AND METHODS

Tissue specimens

Eighteen pairs of human HCC and adjacent non-tumor tissues were obtained from surgical specimens immediately after resection from patients undergoing primary surgical treatment of HCC in the Eastern Hepatobiliary Surgery Hospital, Shanghai, China. No patient had received preoperative irradiation or chemotherapy. The samples were frozen in liquid nitrogen and stored at -80 ℃ until use. Among these samples, three pairs were used for Taqman low-density miRNA array (TLDA) analysis and all were used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Clinical and pathological information was extracted from the patients' medical charts and pathological reports (Table 1). Written consent for tissue donation (for research purposes) was obtained from the patients before tissue collection and the protocol was approved by the Institutional Review Board of Eastern Hepatobiliary Surgery Hospital and Second Military Medical University.

Taqman low-density miRNA array

Total RNA was isolated using mirVana miRNA isolation kit (Ambion, Austin, TX, United States). For miRNA cDNA synthesis, RNA was reversely transcribed using the miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, United States) in combination with the stem-loop Megaplex primer pool (Applied Biosystems). TLDA v2.0 (Applied Biosystems) was performed on the 7900HT real-time PCR system (Applied Biosystems) according to the manufacturer's protocol (667 small RNAs were profiled for each cDNA sample). PCR cycling conditions were as follows: 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. Human U6 small RNA was used as an internal control to normalize RNA input. The data were analyzed using SDS v2.3 software. The Ct value was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method and presented as the fold-expression change in tumors and their adjacent normal tissues after normalization to the endogenous control.

Table 1 Characteristics of patients (n = 18)

No.	Gender	Age (yr)	Tumor size[1] (cm)	Cirrhosis	Tumor grade[2]	TNM stage
1	M	55	1.9	No	G3	T2N0M0
2	M	40	4.1	Macronodular	G3	T2N0M0
3	M	42	4.6	No	G3	T3N0M0
4	M	42	5.6	Micronodular	G2	T3N0M0
5	M	43	5.8	Micronodular	G1	T1N0M0
6	M	61	1.9	Micronodular	G3	T2N0M0
7	M	55	12.0	Micronodular	G2	T4N0M0
8	M	40	4.0	Macronodular	G2	T3N0M0
9	M	35	2.3	Macronodular	G1	T1N0M0
10	M	57	5.9	No	G3	T1N0M0
11	M	44	9.6	No	G2	T3N0M0
12	M	45	12.2	Micronodular	G3	T2N0M0
13	M	46	4.4	Micronodular	G3	T2N0M0
14	F	46	15.8	Micronodular	G3	T2N0M0
15	M	63	6.1	No	G3	T3N0M0
16	M	61	4.6	No	G3	T2N0M0
17	F	60	9.1	No	G3	T3N0M0
18	F	50	9.3	No	G3	T2N0M0

[1]Diameter of the biggest nodule. [2]G1-2: Well-differentiated; G3: Moderately-differentiated; G4: Poorly-differentiated. M: Male; F: Female; TNM: Tumor-node-metastasis.

Quantitative real-time PCR

For miRNA expression analysis, synthesis of cDNA and qRT-PCR was carried out with TaqMan microRNA assay kits (Applied Biosystems) according to the manufacturer's protocol. Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, United States) from HCC and adjacent non-tumor tissues and used to synthesize cDNAs with gene-specific primers. Reverse transcriptase reactions contained 100 ng RNA, 50 nmol/L stem-loop RT primers, 1 × RT buffer, 0.25 mmol/L each of the dNTPs, 3.33 U/μL MultiScribe reverse transcriptase and 0.25 U/μL RNase inhibitor. The 15 μL reactions were incubated for 30 min at 16 ℃, 30 min at 42 ℃, 5 min at 85 ℃, and then kept at 4 ℃. The cDNA product was used for the following qRT-PCR analysis. The 20 μL

PCR reaction included 1.33 μL RT product, 1 × TaqMan universal PCR master mix and 1 μL primers and probe mix of the TaqMan microRNA assay kit. Reactions were incubated in a 96-well optical plate at 95 ℃ for 5 min, followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 1 min. PCR reactions were run on a StepOne Plus real-time PCR machine (Applied Biosystems) and the data were analyzed using SDS v2.3 software, the same as in TLDA.

Prediction of miRNA targets

The target genes of the deregulated miRNAs were predicted by TargetScan (http://www.targetscan.org/).

Gene oncology and Kyoto encyclopedia of genes and genomes pathway analysis based on miRNA expression profile

The miRNA targets were subjected to gene oncology (GO) analysis in order to organize genes into hierarchical categories and uncover the miR-gene regulatory network on the basis of biological process and molecular function[8]. Fisher's exact test and χ^2 test were used to classify the GO category, and the false discovery rate (FDR)[9] was calculated to correct the *P* value, the lower the FDR, the slight the error in judging the *P* value. The FDR was defined as $FDR = 1 - \frac{N_k}{T}$, where N_k refers to the number of Fisher's test *P* values less than χ^2 test *P* values. *P* values were computed for the GOs of all the differential genes. Enrichment provides a measure of the significance of the function: as the enrichment increases, the corresponding function becomes more specific, which can help find those GOs with more concrete function description in the experiment. Within the significant category, the enrichment Re was given by: $Re = (n_f/n) / (N_f/N)$ where n_f is the number of differential genes within the particular category, n is the total number of genes within the same category, N_f is the number of differential genes in the entire array, and N is the total number of genes in the array[10]. Similarly, pathway analysis was used to find out the significant pathway of the differential genes according to Kyoto encyclopedia of genes and genomes (KEGG), Biocarta and Reatome. The Fisher's exact test and χ^2 test were also used to select the significant pathway, and the threshold of significance was defined by *P* value and FDR. The enrichment Re was calculated using the same equation mentioned above[11-13]. The network of miRNA-mRNA interaction, representing the critical miRNAs and their targets, was established according to the miRNA degree.

Construction of luciferase reporter plasmids

The fragment of 3'-untranslated region (UTR) of CCND3 (1054-2061nt, Genbank accession no. NM_001136017.2) containing the two putative miR-138 binding sequences (1279-1285nt and 1346-1352nt) was amplified with the primers 5'-CCCTGGAGAGGCCCTCTGGA-3' and 5'-TTCCAAGAAGCCAAAGCCAG-3'. The partial fragment of 3'-UTR of CHC (5472-6480nt, Genbank accession no. NM_004859) containing the two putative miR-199a-5p binding sequences (5979-5986nt and 5915-5922nt) was amplified with the primers 5'-GATGAAGCGCTGATCCTGTAG-3' and 5'-TGCCTCCCTAATGCCTCAG-3'. The PCR products were cloned into firefly luciferase reporter vector pGL3 (Promega Corporation, Madison, WI, United States) respectively, termed as pGL3-CCND3-3'UTR or pGL3-CHC-3'UTR. The plasmids carrying the mutated sequence in the complementary sites for the seed region of miR-138 or miR-199a-5p, were generated based on pGL3-CCND3-3'UTR and pGL3-CHC-3' UTR plasmids by site-specific mutagenesis, termed as pGL3-CCND3-3'UTR-mut or pGL3-CHC-3'UTR-mut.

Transfection

The transfection was carried out using FuGene HD transfection reagent (Roche, Indianapolis, IN, United States) following the manufacturer's protocol. In brief, 2 × 10^4 HepG2 cells or 5 × 10^4 HEK293T cells in 24-well plate were transfected with indicated miRNA mimic (50 nmol/L, GenePharma, Shanghai, China) or plasmid DNA (100 ng) and collected 24-48 h after transfection for assay.

Dual-luciferase reporter assay

HEK293T cells were cotransfected with pGL3-CCND3-3'UTR or pGL3-CCND3-3'UTR-mut and miR-138 mimic or nonrelative control RNA duplex [non-relative control (NC) duplex, GenePharma] using FuGene HD transfection reagent. In another well, HEK293T cells were cotransfected with pGL3-CHC-3'UTR or pGL3-CHC-3'UTR-mut and miR-199a-5p mimic or nonrelative control RNA duplex using FuGene HD transfection reagent. The pRL-TK (Promega Corporation, Madison, WI, United States) was also transfected as a normalization control. Cells were collected 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay kit (Promega Corporation) and recorded by multi-plate reader (Synergy 2, BioTek).

Western blotting

Protein extracts from HCC tissues and their adjacent non-tumorous tissues or HepG2 cells were prepared by a modified radioimmunoprecipitation buffer with 0.5% sodium dodecyl sulfate in the presence of proteinase inhibitor cocktail (Complete Mini, Roche). Twenty-five micrograms protein were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis mini-gels and transferred onto polyvinylidene fluoride membranes (Immobilon P^{SQ}, Millipore, Billerica, MA, United States). After blocking with 5% nonfat milk, the membranes were incubated with rabbit anti-CCND3 antibody (1:1000 dilution, Epitomics, Inc., Burlingame, CA, United States), rabbit anti-clathrin heavy chain (CHC) antibody (1:1000 dilution, Abcam, Cambridge, United Kingdom) or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:5000 dilution, Epitomics, Inc.) at 4 ℃ overnight, followed by incubation with horserad-

ish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (1:10 000 dilution, KPL, Gaithersburg, MA, United States) for 1h at room temperature. Finally, signals were developed with Super Signal West Pico chemoluminescent substrate (Pierce, Rockford, Ill, United States) and visualized by the Gene Gnome HR Image Capture System (Syngene, Frederick, MD, United States).

Statistical analysis

Data were presented as mean ± SD. Comparisons were made using a two-tailed *t* test or one-way analysis of variance for experiments with more than two subgroups. $P < 0.01$ was considered statistically significant.

RESULTS

Patient characteristics

HCC and adjacent non-tumor tissues were obtained from 18 patients, whose average age was 48.5 years (ranged from 35 years to 71 years) (Table 1). All of the patients were HBsAg positive and with the diagnosis of HCC. In tumor grades, 7 (38.9%) cases were well differentiated (G1 + G2) and 11 (61.1%) cases were moderately differentiated (G3) HCC. In clinical stage, 4 (22.2%) were at invasion T1, 7 (38.9%) at invasion T2, and 6 (33.3%) at invasion T3 and 1 (5.6%) at invasion T4.

Differential expression of miRNAs in HCC

There were 86 deregulated miRNAs in total between HCC tumor and non-tumor tissues by TLDA analysis. In order to select the most significant candidates, miRNAs altered by at least 3-fold in all three pairs of the samples were selected. Under these strict criteria, 11 up-regulated miRNAs and 13 down-regulated miRNAs were identified (Figure 1A and Table 2). To validate the miRNA array data, qRT-PCR was performed in 18 pairs of HCC tissues. Four up-regulated (miR-217, miR-520g, miR-522 and miR-525-3p) (Figure 1B) and 4 down-regulated miRNAs (miR-199a-5p, miR-138, miR-483-5p and miR-511) showed consistent changes in more than 70% tumorous tissues (Figure 1C). Six up-regulated (miR-517c, miR-512-3p, miR-518a-3p, miR-519a, miR-518e and miR-518b) and 7 down-regulated miRNAs (miR-214, miR-214#, miR-592, miR-483-3p, miR-433, miR-708 and miR-1275) showed consistent changes in more than 50% tumorous tissues (data not shown). No significant difference was found between one up-regulated (miR-888) and two down-regulated miRNAs (miR-21# and miR-27a#) in paired tumorous tissues (data not shown).

Gene oncology and Kyoto encyclopedia of genes and genomes pathway analysis of the deregulated miRNAs

The targets of the 21 deregulated miRNAs (Table 2) were predicted by TargetScan. To identify the most significant candidates and investigate the cellular function, the signaling pathway and GOs of the target genes were analyzed. The results showed that a wide variety of cellular processes were featured significantly in signaling pathways (Figure 2A and B). Many of these signaling pathways, such as insulin, MAPK, TGF-β and Wnt signaling pathway, participated in the tumorigenesis[14-17]. However, some other signaling pathways have never been reported to play a role in tumorigenesis, e.g., axon guidance. Among all these differentially regulated signaling pathways, "regulation of actin cytoskeleton" and "pathway in cancer" appeared to be the most enriched one among both up-regulated and down-regulated miRNA groups. A similar phenomenon was observed in GOs analysis. Many cellular functions were featured significantly, of which the "signal transduction" appeared to be the most enriched one (Figure 2C and D). The miRNA-mRNA interaction network analysis integrated these miRNAs and GOs by outlining the interactions of miRNA and GO-related genes (Figure 3A). MiR-519a and miR-199a-5p showed the target genes of 53 (degree 53) in up-regulated miRNAs and target genes of 32 (degree 32) in down-regulated miRNAs. MiR-138 had a degree of 30, which was the second place in down-regulated miRNAs. These results indicated that miR-138 and miR-199a-5p as well as their targets, might be of great importance to the HCC tumorigenesis. Twenty-six target genes, including *RPS6KA3*, *SMAD4*, *ACVR2A*, *CHC*, and *MAPK1*, *etc*., had more than 3 miRNAs (degree > 3) (Figure 3B).

CCND3 is a direct target of miR-138

Among these deregulated miRNAs, miR-138 was most abundant in non-tumor tissues and miR-199a-5p was most significant in paired HCC tissues (Figure 4A). Thus, miR-138 and miR-199a-5p were selected for the further study. CCND3 was predicted as a potential target of miR-138 by TargetScan. The 3'-UTR of CCND3 mRNA contained a complementary site for the seed region of miR-138 (Figure 4B). CCND3 was found to be up-regulated in 4 specimens of HCC tissues compared with adjacent non-tumor liver tissues (Figure 4C), showing a negative correlation with down-regulated miR-138. These results indicated that miR-138 may be associated with CNND3 and both of them may be involved in HCC tumorigenesis.

To validate whether CCND3 is a direct target of miR-138, a human CCND3 3'-UTR fragment containing wild-type or mutant miR-138 binding sequence (Figure 4B) was cloned downstream of the firefly luciferase reporter gene in pGL3. In HEK293 cells cotransfected with the reporter plasmids and miR-138 mimic or NC duplex, the luciferase activity of the reporter that contained wild-type 3'-UTR was significantly suppressed by miR-138 mimic, but the luciferase activity of mutant reporter was unaffected (Figure 4D), indicating that miR-138 may suppress gene expression through miR-138 binding sequence at the 3'-UTR of CCND3. Furthermore, transfection of miR-138 mimic decreased CCND3 expression in HepG2 cells at protein level (Figure 4E). All these results showed that miR-138 could regulate the expression of endogenous human CCND3 by directly

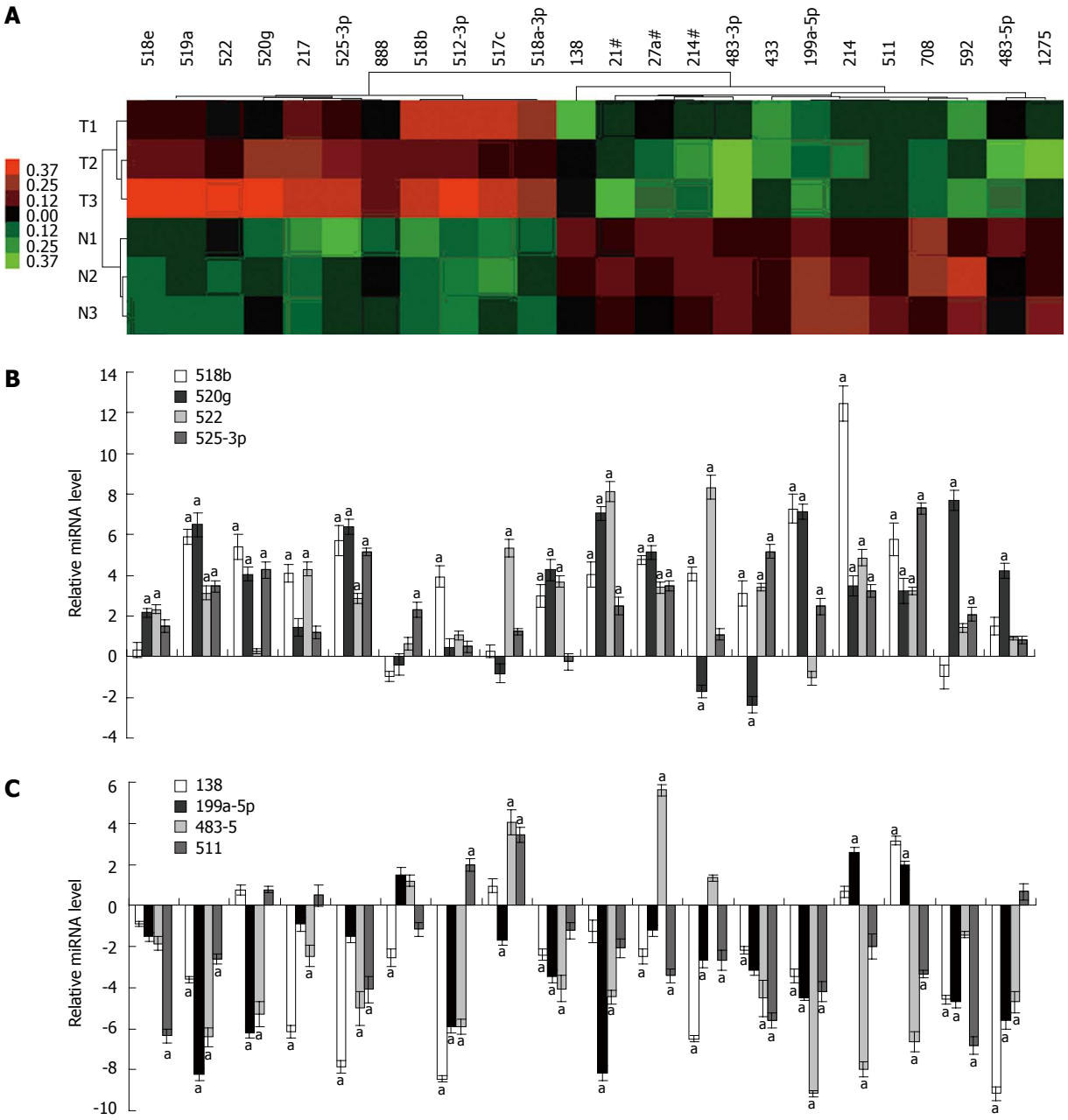


Figure 1 MiRNA profiles differentiate hepatitis B virus-associated hepatocellular carcinoma from adjacent non-tumor tissues. A: The cluster analysis of down-regulated (green) and up-regulated (red) miRNAs identified in hepatocellular carcinoma (hepatitis B virus-hepatocellular carcinoma). Samples consist of paired samples from three patients; B: Validation of Taqman array data using quantitative reverse transcription polymerase chain reaction (RT-PCR) for up-regulated miRNA; C: Validation of Taqman array data using quantitative RT-PCR for down-regulated miRNA. Triplicate assays were done for each RNA sample and the relative amount of each miRNA was normalized to U6 snRNA. $^{a}P < 0.01$ *vs* control group.

targeting the 3'-UTR of CCND3 mRNA and human CCND3 was a new target of miR-138.

Clathrin heavy chain is a direct target of miR-199a-5p

CHC was found to be a direct target of miR-199a-5p. CHC was predicted as a potential target of miR-199a-5p by TargetScan (Figure 4B). CHC was up-regulated in 4 specimens of HCC tissues as compared with adjacent non-tumor liver tissues (Figure 4C). The luciferase activity of the reporter containing wild-type 3'-UTR of CHC was significantly suppressed by miR-199a-5p mimic (Figure 4D). Furthermore, transfection of miR-199a-5p mimic decreased CHC expression in HepG2 cells at protein level (Figure 4E). The results showed that miR-199a-5p could regulate the expression of endogenous human CHC by directly targeting the 3'-UTR of CHC mRNA, and human CHC is a new target of miR-199a-5p.

DISCUSSION

MiRNAs were frequently deregulated in HCC, and some specific miRNAs were associated with the clinicopatho-

Table 2 Deregulated miRNA in hepatitis B virus-associated hepatocellular carcinoma *n* (%)

miRNA	Fold change	*P* value	Validation[1]
Up-regulated miRNAs			
hsa-miR-520g	9475.09	0.00	13 (72.2)
hsa-miR-519a	8204.48	0.00	10 (55.6)
hsa-miR-522	6178.34	0.00	13 (72.2)
hsa-miR-518e	4096.74	0.00	11 (61.1)
hsa-miR-525-3p	1489.49	0.00	14 (77.8)
hsa-miR-217	1080.33	0.00	11 (59.4)
hsa-miR-518b	982.85	0.00	14 (77.8)
hsa-miR-512-3p	197.21	0.00	10 (55.6)
hsa-miR-517c	130.92	0.00	11 (61.1)
hsa-miR-518a-3p	46.84	0.00	10 (55.6)
Down-regulated miRNAs			
hsa-miR-199a-5p	0.00237	0.00050	13 (72.2)
hsa-miR-433	0.00310	0.00270	10 (55.6)
hsa-miR-592	0.01137	0.00350	11 (59.4)
hsa-miR-214#	0.01327	0.00610	11 (59.4)
hsa-miR-483-5p	0.03310	0.00340	14 (77.8)
hsa-miR-483-3p	0.03323	0.00510	12 (72.2)
hsa-miR-138	0.03727	0.00190	14 (77.8)
hsa-miR-214	0.04213	0.00530	11 (59.4)
hsa-miR-511	0.07957	0.00490	13 (72.2)
hsa-miR-708	0.02135	0.00480	10 (55.6)
hsa-miR-1275	0.06296	0.00530	10 (55.6)

[1]Quantitative reverse-transcription polymerase chain reaction was performed in 18 pairs of tumor and non-tumor tissues in hepatitis B virus-associated hepatocellular carcinoma.

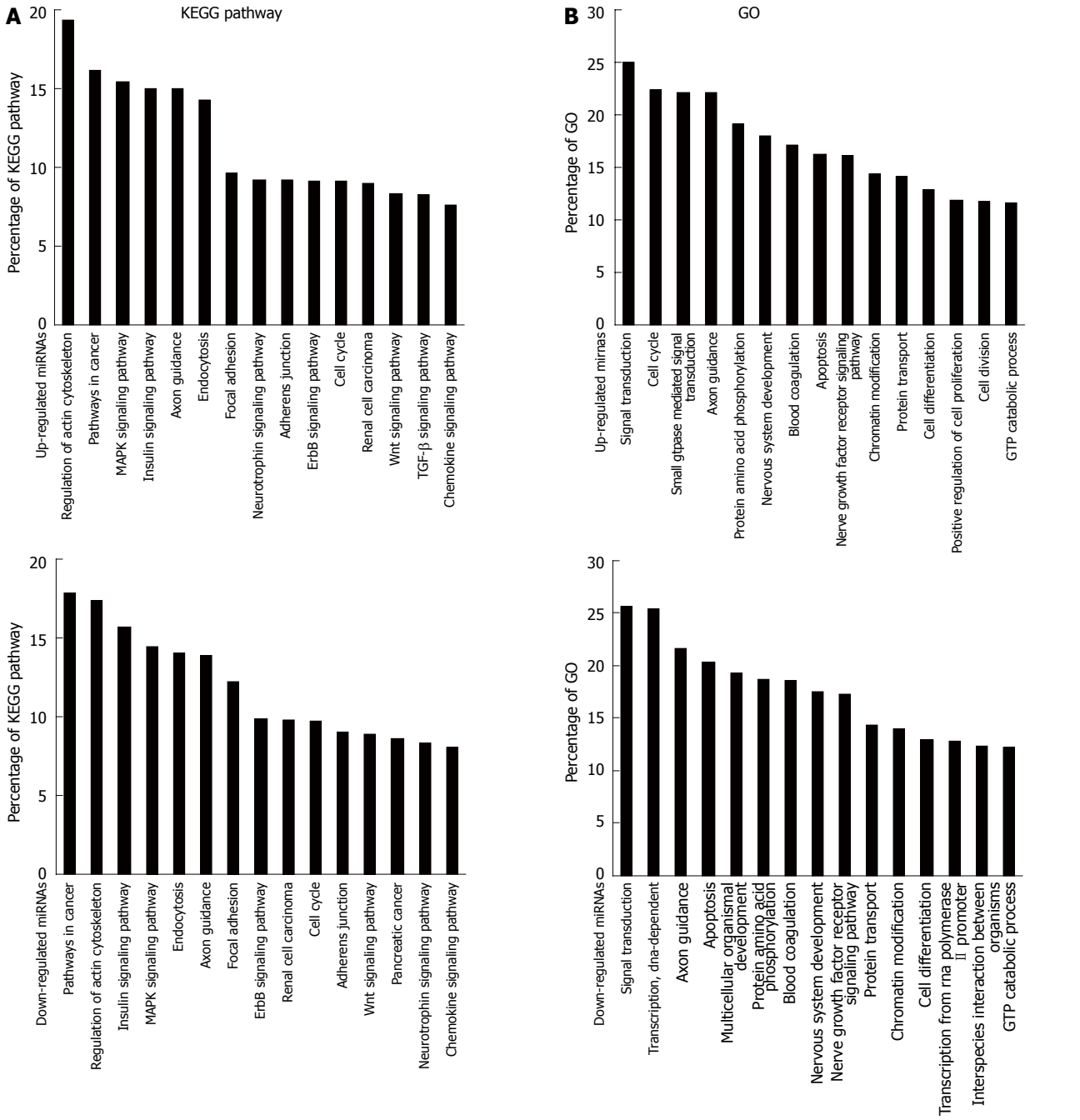
logical features of HCC, such as metastasis, recurrence, and prognosis[18-20]. Moreover, compelling evidence has demonstrated that miRNAs play an important role in HCC progression and directly contribute to the cell proliferation, avoidance of apoptosis, and metastasis of HCC. Identifying the miRNAs and their targets that are essential for HCC progression may provide promising therapeutic opportunities.

In this study, with Taqman miRNAs array and real-time RT-PCR confirmation, 10 up-regulated miRNAs (miR-217, miR-518b, miR-517c, miR-520g, miR-519a, miR-522, miR-518e, miR-525-3p, miR-512-3p, and miR-518a-3p) and 11 down-regulated miRNAs (miR-138, miR-214, miR-214#, miR-199a-5p, miR-433, miR-511, miR-592, miR-483-5p, miR-483-3p, miRNA-708 and miRNA-1275) were identified in HCC. More importantly, of these 21 deregulated miRNAs, only miR-199a-5p was involved in HCC[21,22], and the other 20 deregulated miRNAs were first reported to be involved in HCC tumorigenesis. No report of MiR-214#, miR-518a-3p and miR-518e has been available in the literature. The rest 17 miRNAs were reported in various cancer but not HCC. For example, the up-regulated miR-512-3p and miR-525-3p were associated with a cisplatin resistant phenotype in human germ cell tumors[23]. The up-regulated miR-519a and down-regulated miR-511 and miR-485-5p were associated with histological subtypes in ovarian cancers[24]. MiR-517c and 520 g promote *in vitro* and *in vivo* oncogenicity, modulates cell survival, and robustly enhances growth of untransformed human neural stem cells (hNSCs) in neuroectodermal brain tumors[25]. MiR-433 could regulate tumor-associated proteins GRB2 in gastric carcinoma[26]. MiR-592 was reported to be associated with the stepwise progression for transformation from normal colon to carcinoma[27].

On the contrary, some well known HCC-related miRNAs were not found in this study, possibly due to the very strict criteria of selection defined in TLDA (significant difference in all three pairs of HCC tissues). For example, miR-21[28], miR-122[29,30], miR-16[31] and miR-29[32,33] were excluded for their significantly differential expression only in 2 pairs of HCC tissues, and miR-181[34], miR-221[35,36], miR-125[37] and miR-101[38] were excluded for their differential expression in only 1 pair of HCC tissues or no differential expression at all. Using such strict criteria, we might miss some important candidates, but catch some unique ones. Fortunately, quantitative RT-PCR and follow-up studies proved that our strategy helped find the above new deregulated miRNAs. All of the HCC patients in this study were HBsAg positive. Therefore, the expression patterns of identified miRNAs may mainly represent the alterations in hepatitis B virus (HBV)-positive HCC, which may partially account for the inconsistency between our results and results from other studies.

KEGG pathway and GO enrichment analysis based on the reported and predicted target genes of these deregulated miRNAs, was applied to identify which particular functions and pathways were enriched among the genes controlling distinctive characters between HCC and adjacent non-tumor tissues. As a result, KEGG pathway analysis showed that proliferative (cell cycle, MAPK and Wnt), adhesive (actin cytoskeleton, adherens junction and focal adhesion), survival (TGF-β and ErbB) and oncogenic (renal cell carcinoma and pancreatic cancer) signaling pathways were abundant among the significantly enriched ones. Furthermore, the GOs related to signal transduction (signal transduction, small GTPase-mediated signal transduction, protein amino acid phosphorylation and nerve growth factor receptor signaling pathway) and cell growth (cell differentiation, cell division, positive regulation of cell proliferation, cell cycle and multicellular organism development) represented up to 37% of the significantly enriched GOs. As expected, various cell process and signal pathways were involved in HCC tumorigenesis. To narrow the scope of study and evaluate the most significant candidates, miRNAs and their target genes which were in the intersection of "signal transduction" and "regulation of actin cytoskeleton and pathway in cancer" might be the focus of the future studies.

Although computational analysis indicates that one miRNA may directly modulate hundreds of mRNAs, and a single gene may be regulated by multiple miRNAs, such regulation has not been convincingly demonstrated experimentally. As shown in Figure 3, miR-138 had 30 target genes and miR-199a-5p had 32 target genes. The



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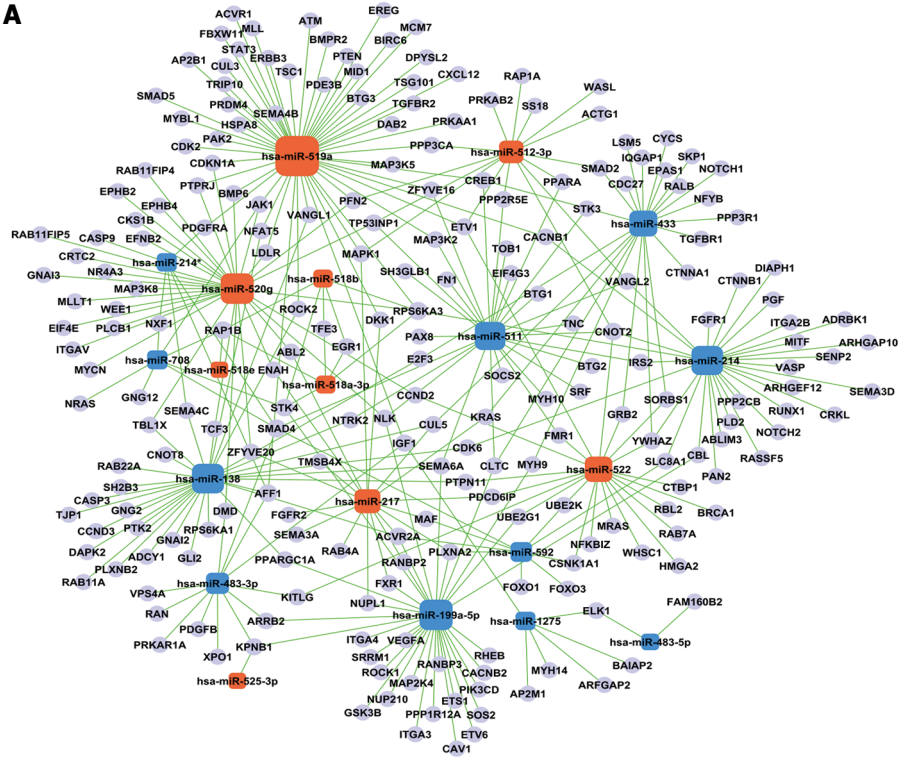
	Significant pathways	Rank	
		Up-regulated	Down-regulated
1	Regulation of cytoskeleton	1	2
2	Pathways in cancer	2	1
3	MAPK signaling pathway	3	4
4	Insulin signaling pathway	4	3
5	Axon guidance	5	6
6	Endocytosis	6	5
7	Focal adhesion	7	7
8	Neurotrophin signaling pathway	8	14
9	Adherens junction	9	11
10	ErbB signaling pathway	10	8
11	Cell cycle	11	10
12	Renal cell carcinoma	12	9
13	Wnt signaling pathway	13	12
14	Chemokine signaling pathway	15	15

D

	Significant GOs	Rank	
		Up-regulated	Down-regulated
1	Signal transduction	1	1
2	Axon guidance	4	3
3	Protein anima acid phosphorylation	5	6
4	Apoptosis	8	4
5	Blood coagulation	7	7
6	Nerve growth factor receptor signaling pathway	9	9
7	Chromatin modification	10	11
8	Protein transport	11	10
9	GTP catabolic process	15	15

Figure 2 Gene oncology and kyoto encyclopedia of genes and genomes pathway analysis based on miRNA targeted genes. A: The upper panel showing significant pathways targeted by up-regulated miRNA and the lower panel showing significant pathways targeted by down-regulated miRNA; B: The upper panel showing significant GOs targeted by up-regulated miRNA and the lower panel showing significant GOs targeted by down-regulated miRNA. The vertical axis is the pathway or GO category, and the horizontal axis is the enrichment of pathways or GOs; C, D: Summary data of A and B respectively. KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene ontology.

A



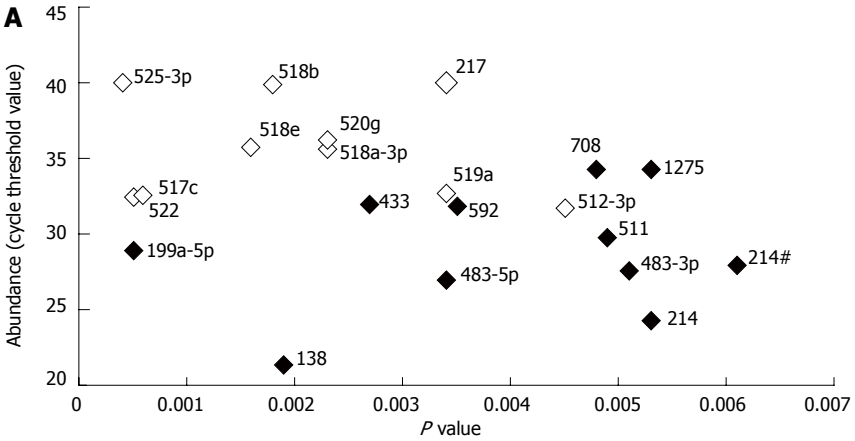
B

Up-regulated miRNA (top 3)	Degree	Down-regulated miRNA (top 3)	Degree
has-miR-519a	53	has-miR-199a-5p	32
has-miR-520g	32	has-miR-138	30
has-miR-522	21	has-miR-214	29

Gene symbol	Degree	Gene symbol	Degree
RPS6KA3	6	LDLR	3
SMAD4	5	MAPK1	3
ACVR2A	4	NLK	3
AFF1	4	PEN2	3
CNOT2	4	PPP2R5E	3
PLXNA2	4	PTPN11	3
RAP1B	4	SEMA6A	3
CHC	3	SLC8A1	3
CREB1	3	STK4	3
CUL5	3	TP53INP1	3
DMD	3	VANGL1	3
ETV1	3	VANGL2	3
KPNB1	3	YWHAZ	3

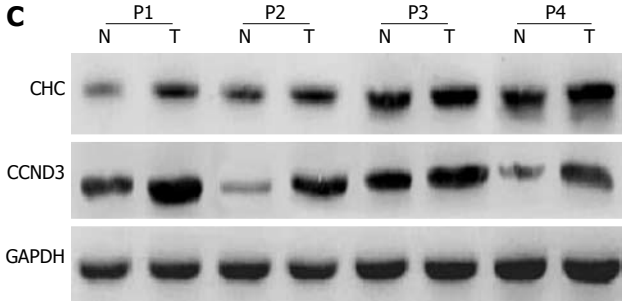
Figure 3 MiRNAs-mRNA network. A: Orange box nodes represent up-regulated miRNAs, blue box nodes represent down-regulated miRNAs, and cyan cycle nodes represent mRNA. Green lines show the inhibitory effect of miRNAs on target mRNAs; B: Summary data of A.

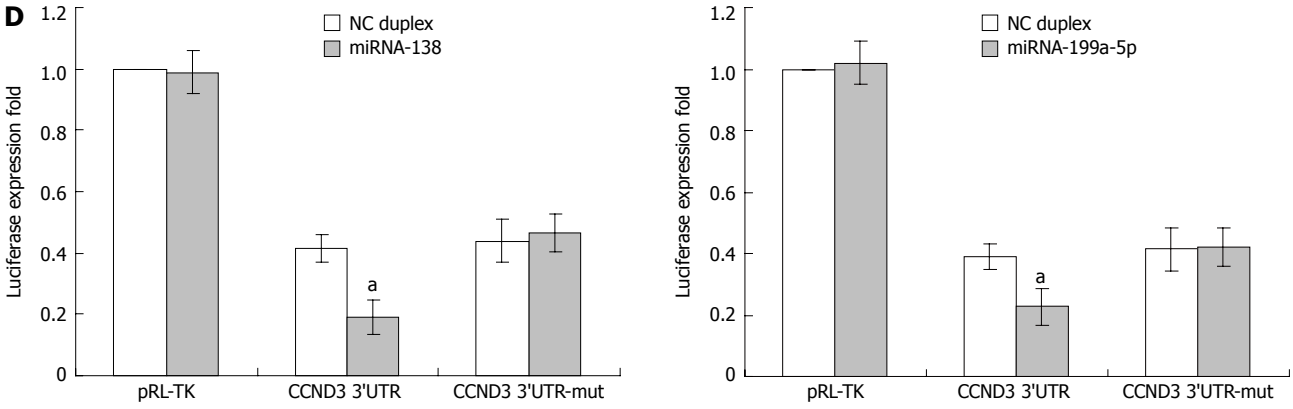
target genes are involved in different cellular processes, thus individual miRNAs play multi-faceted roles in HCC progression. Down-regulated miR-138 has been observed in different types of cancers but not in HCC[39-43]. MiR-138 plays an important role in tongue squamous cell carcinoma cell migration and invasion by concurrently targeting RhoC and ROCK2[39]. MiR-138 could inhibit the expression of HIF-1a and regulate the apoptosis and migration of clear-cell renal cell carcinoma 786-O cells[40]. MiR-138 enhanced cell migration and invasion by targeting enhancer of zeste homologue 2 (EZH2) in squamous cell carcinoma cell lines[41]. MiR-138 may play an important role in cancer initiation and progression by regulating Fos-like antigen 1 in squamous cell carcinoma[42] or G protein alpha inhibiting activity polypeptide 2(GNAI2) in tongue squamous cell carcinoma[43]. Different from miR-138, the down-regulated miR-199a-5p has been reported in HCC[21,22]. MiR-199a-5p contributes to the increase of cell invasion by functional deregulation of discoidin domain receptor-1 (DDR1) activity in HCC[21]. MiR-199a-5p regulates Brm subunit of SWI/SNF in human cancers[22]. We identified CCND3 as a target of miR-138 and CHC as a target of miR-199a-5p in HCC, which may provide new insights into the mechanisms underlying tumorigenesis. CCND3 is expressed in nearly all proliferating cells and can promote the cell cycle progres-



B

	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 292-298 of CCND3 3' UTR	5'...AGAAUUGGAUACAUACACCAGCA...
has-miR-138	3' GCCGGACUAAGUGUUGUGGUCGA
Mut CCND3 3'UTR	5'...AGAAUUGGAUACAUAGUGGUCGA...
Position 225-231 of CCND3 3' UTR	5'...UCCUCCCUGCAUCUGACCAGCA...
has-miR-138	3' CCGGACUAAGUGUUGUGGUCGA
Mut CCND3 3'UTR	5'...UCCUCCCUGCAUCUGUGGUCGA...
Position 508-515 of CHC 3' UTR	5'...UAAUCAGCACAUCGUACACUGGA...
has-miR-199a-5p	3' CUUGUCCAUCAGACUUGUGACCC
Mut CCND3 3'UTR	5'...UAAUCAGCACAUCGUUGUGACCA...
Position 444-451 of CHC 3' UTR	5'...AAAGCUAAAGCAAAAACACUGGC...
has-miR-199a-5p	3' CUUGUCCAUCAGACUUGUGACCC
Mut CCND3 3'UTR	5'...AAAGCUAAAGCAAAAUGUGACCC...





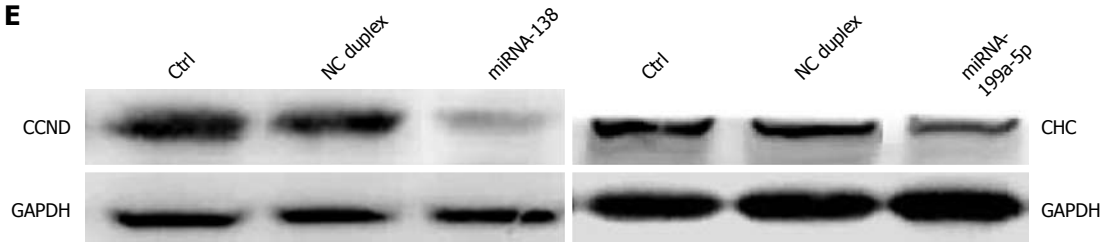


Figure 4 Cyclin D3 and clathrin heavy chain are the direct target of miR-138 and miR-199a-5p. A: Abundance of deregulated miRNAs in hepatitis B virus (HBV)-hepatocellular carcinoma (HCC) non-tumor tissues; B: The putative miR-138 or miR-199a-5p binding sequence in the 3'-UTR of cyclin D3 (CCND3) or clathrin heavy chain (CHC) mRNA; C: The expression of CCND3 and CHC in 4 paired HCCs (T) and adjacent non-tumor tissues (N); D: Suppressed luciferase activity of wild type 3'UTR of CCND3 or CHC by miR-138 or miR-199a-5p mimic. Firefly luciferase activity of each sample was mea sured 48 h after transfection and normalized to Renilla luciferase activity; E: Suppressed expression of endogenous CCND3 or CHC in HepG2 cells by miR-138 or miR-199a-5p mimic, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Column, mean of three independent experiments; bars, SD; $^{a}P < 0.01$ *vs* control group. NC: Non-relative control.

sion[44,45]. Liu Q reported that miR-16 family (including miR-16, miR-195 and miR-424) could induce cell cycle arrest by targeting CCND3, CCNE1 and Cdk6[46]. CHC is a part of clathrin expressed ubiquitously and exerts important functions in endocytosis and mitosis[47]. CHC has been reported to play an important role in inflammation disorder and tumorigenesis[48-51]. CHC functions as a co-activator for p53[48] and contributes to the regulation of basal NF-κB activity in epithelial cells[49]. CHC was identified as immunohistochemical tumor markers for primary HCC[50] and served as a biomarker for early diagnosis of small HCC[51]. Whether CCND3 and CHC regulated by miR-138 and miR-199a-5p respectively are involved in HCC tumorigenesis or other cell functions needs further studies.

In summary, we reported 21 deregulated miRNAs in HCC and CCND3 as the target of miR-138 and CHC as the target of miR-199a-5p. Our findings indicated that the novel miRNAs might be involved in HCC tumorigenesis and provide more evidence for the reliability of integrative bioinformatics analysis.

COMMENTS

Background

MiRNAs are endogenous non-coding RNAs (20-22 nucleotides) that have been identified as post-transcriptional regulators of gene expression. The miRNAs mainly bind to the 3' untranslated regions (UTRs) of target mRNAs, resulting in mRNA degradation or the blockade of mRNA translation. Increasing evidence has demonstrated that miRNAs play an important role in hepatocellular carcinoma (HCC) progression and directly contribute to the cell proliferation, avoidance of apoptosis, and metastasis of HCC. Identifying the miRNAs and their targets that are essential for HCC progression may provide promising therapeutic opportunities.

Research frontiers

Most recently identified miRNAs were found to be frequently deregulated in HCC, and some specific miRNAs were found to be associated with the clinicopathological features of HCC, such as metastasis, recurrence, and prognosis. Moreover, compelling evidence has demonstrated that miRNAs play an important role in HCC progression and directly contribute to the cell proliferation, avoidance of apoptosis, and metastasis of HCC.

Innovations and breakthroughs

In this study, 10 up-regulated miRNAs (miR-217, miR-518b, miR-517c, miR-520g, miR-519a, miR-522, miR-518e, miR-525-3p, miR-512-3p, and miR-518a-3p) and 10 down-regulated miRNAs (miR-138, miR-214, miR-214#, miR-27a#, miR-199a-5p, miR-433, miR-511, miR-592, miR-483-5p and miR-483-3p) were identified in HCC. Of the 20 deregulated miRNAs, only miR-199a-5p was reported to contribute to the increase of cell invasion by functional deregulation of discoidin domain receptor-1 activity in HCC and regulate Brm subunit of SWI/SNF in human cancers, and the other 19 deregulated miRNAs were first reported to be involved in HCC tumorigenesis. MiR-27a#, miR-214#, miR-518a-3p and miR-518e have never been reported in literatures. The rest 15 miRNAs were reported in various cancers, but not in HCC. Compared with the previous version, the newly found miRNAs (337 updated miRNAs) were profiled in Taqman low-density miRNA array v2.0, which helped the authors identify many new differentially expressed miRNAs in this study.

Applications

This study provides new insights into the understanding of the molecular mechanisms of hepatic carcinogenesis regulated by miRNA, and helps develop personalized miRNA-based therapeutics against HCC.

Terminology

MiRNAs are endogenous non-coding 20 to 22 nucleotide RNAs that have been identified as post-transcriptional regulators of gene expression. MiRNAs are processed from precursor molecules (pri-miRNAs), which are either transcribed from independent miRNA genes or are portions of introns of protein-coding RNA polymerase Ⅱ transcripts. A single pri-miRNA often contains sequences of several different miRNAs. Pri-miRNAs fold into hairpin structures containing imperfectly base-paired stems and are processed in two steps, catalyzed by the RNase III type endonucleases Drosha (also known as RN3) and Dicer. The Drosha-DGCR8 complex processes pri-miRNAs to ~70-nucleotide hairpins known as pre-miRNAs. In animals, premiRNAs are transported to the cytoplasm by exportin5, where they are cleaved by Dicer to yield ~20-bp miRNA duplexes. One strand is then selected to function as a mature miRNA, while the other strand is degraded. Occasionally, both arms of the pre-miRNA hairpin give rise to mature miRNAs.

Peer review

This article aimed to reveal microRNAs and target genes associated with hepatocellular carcinogenesis using miRNA array and network/pathway analyses combined with integrative bioinformatical analysis. The topic is of significant clinical importance as HCC is a very common and usually lethal liver tumor, and all researches that may reveal some potential target genes/miRNAs that offer new therapeutic possibilities are useful for the better understanding of the disease and related molecular biological mechanisms.

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BRIEF ARTICLE

Correlation between circulating myeloid-derived suppressor cells and Th17 cells in esophageal cancer

Zhi-Jun Jiao, Jing-Jing Gao, Sheng-Hao Hua, De-Yu Chen, Wen-Hong Wang, Hui Wang, Xu-Hui Wang, Hua-Xi Xu

Zhi-Jun Jiao, Jing-Jing Gao, Sheng-Hao Hua, Hui Wang, Xu-Hui Wang, Key Laboratory of Medical Immunology, Department of Laboratory Medicine, Affiliated Hospital of Jiangsu University, Zhenjiang 212013, Jiangsu Province, China
De-Yu Chen, Institute of Oncology, Affiliated Hospital of Jiangsu University, Zhenjiang 212001, Jiangsu Province, China
Wen-Hong Wang, Hua-Xi Xu, Department of Pathogenic Biology, School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang 212013, Jiangsu Province, China
Author contributions: Jiao ZJ, Xu HX and Wang WH designed the research; Chen DY collected the clinical data; Gao JJ, Hua SH, Wang H and Wang XH contributed to the acquisition of data or to the analysis and interpretation of data; Jiao ZJ and Gao JJ wrote the paper.
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Correspondence to: Dr. Zhi-Jun Jiao, Key Laboratory of Medical Immunology, Department of Laboratory Medicine, Affiliated Hospital of Jiangsu University, No. 438, Jiefang Road, Zhenjiang 212001, Jiangsu Province, China. jiaozhijun@yahoo.com.cn
Telephone: +86-511-85021135 Fax: +86-511-85029089
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Abstract

AIM: To perform a comprehensive investigation into the potential correlation between circulating myeloid-derived suppressor cells (MDSCs) and Th17 cells in esophageal cancer (ECA).

METHODS: A total of 31 patients newly diagnosed with ECA and 26 healthy subjects were included in the current study. The frequencies of MDSCs and Th17 cells in peripheral blood were determined by flow cytometry. The mRNA expression of cytokines, arginase 1 (Arg1) and inducible NO synthase (iNOS) in peripheral blood mononuclear cells (PBMCs) and plasma Arg1 were assessed by real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively.

RESULTS: There was an increased prevalence of MDSCs in the peripheral blood from ECA patients (15.21% ± 2.25%) when compared with healthy control (HC) (1.10% ± 0.12%, $P < 0.0001$). The plasma levels of Arg1 in ECA patients were significantly higher than those in HC (28.28 ± 4.10 ng/mL *vs* 9.57 ± 1.51 ng/mL, $P = 0.0003$). iNOS mRNA levels in the peripheral blood of ECA patients also showed a threefold increase compared with HC ($P = 0.0162$). The frequencies of Th17 cells ($CD4^+IL\text{-}17A^+$) were significantly elevated in ECA patients versus HC (3.50% ± 0.33% *vs* 1.82% ± 0.19%, $P = 0.0001$). Increased mRNA expression of IL-17 and ROR-γt was also observed in ECA patients compared with HC ($P = 0.0041$ and $P = 0.0004$, respectively), while the mRNA expression of IL-6 and tumor necrosis factor-α (TNF-α) showed significant decreases ($P = 0.0049$ and $P < 0.0001$, respectively). No obvious correlations were found between the frequencies of MDSCs and Th17 cells in the peripheral blood from ECA patients($r = -0.1725$, $P = 0.3534$). Arg1 mRNA levels were positively correlated with levels of IL-6 ($r = 0.6404$, $P = 0.0031$) and TNF-α ($r = 0.7646$, $P = 0.0001$). Similarly, iNOS mRNA levels were also positively correlated with levels of IL-6 ($r = 0.6782$, $P = 0.0007$) and TNF-α ($r = 0.7633$, $P < 0.0001$).

CONCLUSION: This study reveals the relationship between circulating MDSCs and Th17 cells, which may lead to new immunotherapy approaches for ECA based on the associated metabolites and cytokines.

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Key words: Myeloid-derived suppressor cells; Th17 cells; Esophageal cancer; Arginase Ⅰ; Peripheral blood mononuclear cells; Inducible NO synthase

Peer reviewer: Dr. Satoru Motoyama, Department of Surgery, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan

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INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of cells comprised of myeloid progenitor cells and immature myeloid cells, which exert suppressive functions, regulating T cell responses through the production of arginase 1 (Arg1), nitric oxide and reactive oxygen species. These cells are suspected to play a crucial role in local and systemic tumor development, providing a beneficial microenvironment in which tumor cells can proliferate, expand, acquire new mutations and escape host immunosurveillance[1]. Elevated numbers of MDSCs in peripheral blood have been demonstrated in a substantial number of studies in different types of cancer, including malignant gliomas[2], head and neck cancer[3,4], invasive breast carcinomas[4], colon carcinoma[5], pancreatic cancer[6] and mesothelioma[7].

In esophageal cancer (ECA), it has recently been shown that there is a significant increase in the levels of circulating MDSCs, which is correlated with elevated numbers of regulatory T cells (Tregs) and associated with an increased expression of Arg1[6]. A recent report has also identified that MDSCs not only modulate the de novo induction of Tregs and Th17 cells from $CD4^+$ T cells but also catalyze the transdifferentiation of $Foxp3^+$ regulatory T cells from monocyte-induced Th17 cells[8]. These findings suggested that the interaction between MDSCs and T cell subsets may play an important role in the balance of anti- and pro-tumor immune responses. As a new member of the $CD4^+$T-cell family, Th17 cells have been characterized as preferential producers of interleukin (IL)-17A, IL-17F, IL-21, IL-22, and tumor necrosis factor-α (TNF-α) and have been found in increased numbers in tumor-bearing hosts, including ECA[9-11]. However, it is still controversial whether these Th17 cells promote or inhibit tumor progression[12-14]. Furthermore, whether there is a correlation between circulating MDSCs and Th17 cells in ECA remains unclear. The present study was designed to evaluate the relationship between circulating MDSCs and Th17 cells by examining the cell frequencies and related cytokines and other associated products in the peripheral blood from patients with ECA.

MATERIALS AND METHODS

Patients and healthy controls

Thirty-one patients (25 male, 6 female) newly diagnosed with ECA were included in the current study. These patients ranged from 50 to 78 years of age (average age, 61.97 ± 1.24 years). Twenty-six healthy subjects, matched for age and sex with the ECA patients, were studied as the controls. No subject was treated preoperatively or had a history of autoimmune disease, and no healthy control had a prior history of cancer. This study was approved by the research ethics committee of the Affiliated Hospital of Jiangsu University, and written informed consent was obtained from all individuals.

Table 1 Primer pairs used in real-time polymerase chain reaction analysis

Target cDNA	Upper/lower	Sequence (5′ to 3′)
IL-17	U	CAGATTACTACAACCGATCC
	L	ATGTGGTAGTCCACGTTCC
ROR-γt	U	GTGCTGGTTAGGATGTGCCG
	L	GTGGGAGAAGTCAAAGATGGA
IL-6	U	AAAGAGGCACTGGCAGAAAA
	L	TTTCACCAGGCAAGTCTCCT
TNF-α	U	TAGCCCATGTTGTAGCAAACC
	L	ATGAGGTACAGGCCCTCTGAT
Arg1	U	CAAGAAGAACGGAAGAATCAGC
	L	TTGTGGTTGTCAGTGGAGTGTT
iNOS	U	CTTTCCAAGACACACTTCACCA
	L	TATCTCCTTTGTTACCGCTTCC
β-actin	U	TGGCACCCAGCACAATGAA
	L	CTAAGTCATAGTCCGCCTAGAAGCA

TNF-α: Tumor necrosis factor-α; IL: Interleukin; iNOS: Inducible NO synthase; Arg: Arginase; U: Upper; L: Lower.

Flow cytometric quantification of myeloid-derived suppressor cells

The MDSC population was defined as $HLA\text{-}DR^-/CD14^-/CD33^+/CD11b^+$. Heparinized venous blood was freshly obtained from either the ECA patients or the healthy donors. One hundred microliters of blood was mixed with 5μl of each antibody (BD Bioscience, San Jose, CA or eBioscience, San Diego, CA, United States), then incubated in a dark room for 15 min. Each sample was then mixed with 1 mL of 1 × lysing buffer (BD Biosciences). After incubation, the samples were washed with phosphate buffered saline (PBS), and the pellets were resuspended in 250 μL of PBS. Labeled cells were washed and analyzed with a FACSCalibur flow cytometer (Becton-Dickinson) using CellQuest software (Becton-Dickinson). In each case, staining was compared with that of the appropriately labeled isotype control antibody.

Flow cytometric quantification of Th17 cells

Following centrifugation, the plasma was removed and stored at -70 ℃, while peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation. PBMCs were used in two parts. Some PBMCs were frozen at -70 ℃ after mixing with TRIzol (Invitrogen, Carlsbad, CA, United States) for extracting total RNA, while the remaining cells was used for analysis of Th17 cells. Briefly, PBMCs were stimulated for 5 h using 50 ng/mL of phorbol myristate acetate (PMA, Sigma-Aldrich, MO, United States) and 1 g/mL ionomycin (Sigma-Aldrich, MO, United States) in the presence of 5 g/mL brefeldin A (Sigma-Aldrich, MO, United States) at

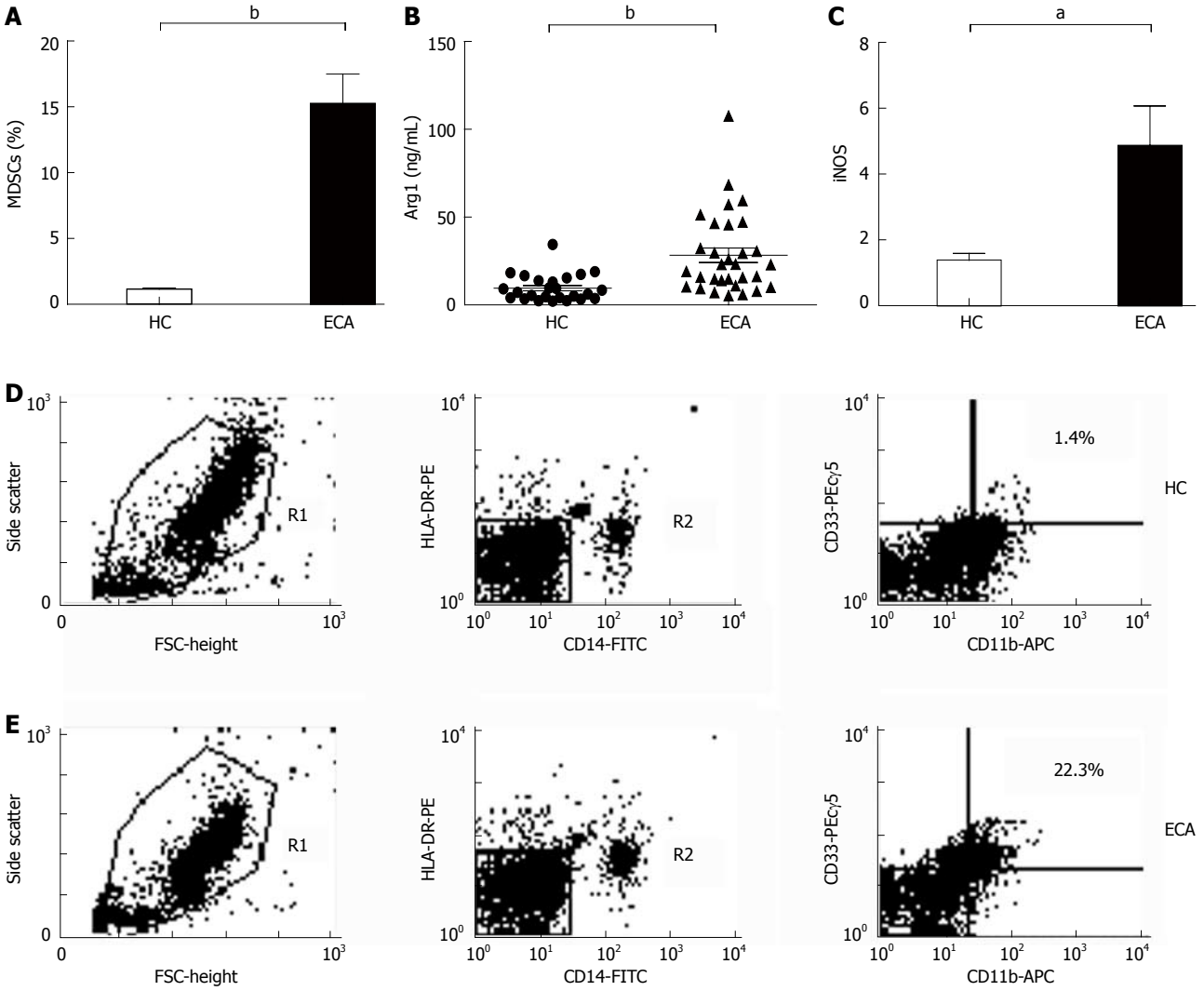


Figure 1 Elevated levels of myeloid-derived suppressor cells and their products, arginase 1 and inducible NO synthase, in esophageal cancer compared with healthy controls. A: Flow cytometric analysis of MDSCs in the peripheral blood from ECA patients and healthy control (HC); B: Plasma arginase 1 (Arg1) level was determined by ELISA; C: The mRNA level of iNOS in the peripheral blood from ECA patients and HC were assessed by real-time PCR; D: Representative flow diagrams of a healthy volunteer with marked elevation of circulating MDSCs; E: Representative flow diagrams of an ECA patient with marked elevation of circulating MDSCs are shown. Data were analyzed by the Student's *t*-test. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* HC group. ECA: Esophageal cancer; MDSCs: Myeloid-derived suppressor cells; ELISA: Enzyme linked immunosorbent assay; iNOS: Inducible NO synthase; PCR: Polymerase chain reaction; FITC: Fluorescein isothiocyanate; FSC: Forward scatter; HLA-DR-PE: HLA-DR-phycoerythrin; APC: Allophycocyanin.

37 ℃ and 5% CO_2. The cells were then washed in PBS and surface-labeled with CD4-FITC (eBioscience, San Diego, CA, United States). Following surface staining, the cells were fixed and permeabilized using IntraPrep Permeabilization Reagent (Beckman Coulter Inc., Fullerton, CA, United States) and then stained with IL-17A-PE (eBioscience, San Diego, CA, United States)[11]. Labeled cells were washed and analyzed with a FACSCalibur flow cytometer (Becton-Dickinson) using the CellQuest software (Becton-Dickinson).

RNA extraction, cDNA synthesis and polymerase chain reaction

Total RNA was extracted from individual PBMC preparations using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States). cDNA was prepared by reverse transcription with oligo (dT) from total RNA extraction. Real-time polymerase chain reaction (PCR) for IL-17, IL-6, TNF-α, Arg1, iNOS and a reference gene (β-actin) was performed in a LightCycler instrument (Roche Molecular Diagnostics, Mannheim, Germany) using the SYBRgreen mastermix kit (TaKaRa, Ohtsu, Japan). The expression data for each target gene were then normalized relative to β-actin. All primer sequences are shown in Table 1.

Enzyme-linked immunosorbent assay

Plasma levels of Arg1 were measured using an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocols (eBioscience, San Diego, CA, United States). Hemolyzed samples were excluded. All samples were assayed in triplicate, and the mean absorbance was calculated from the standard curve.

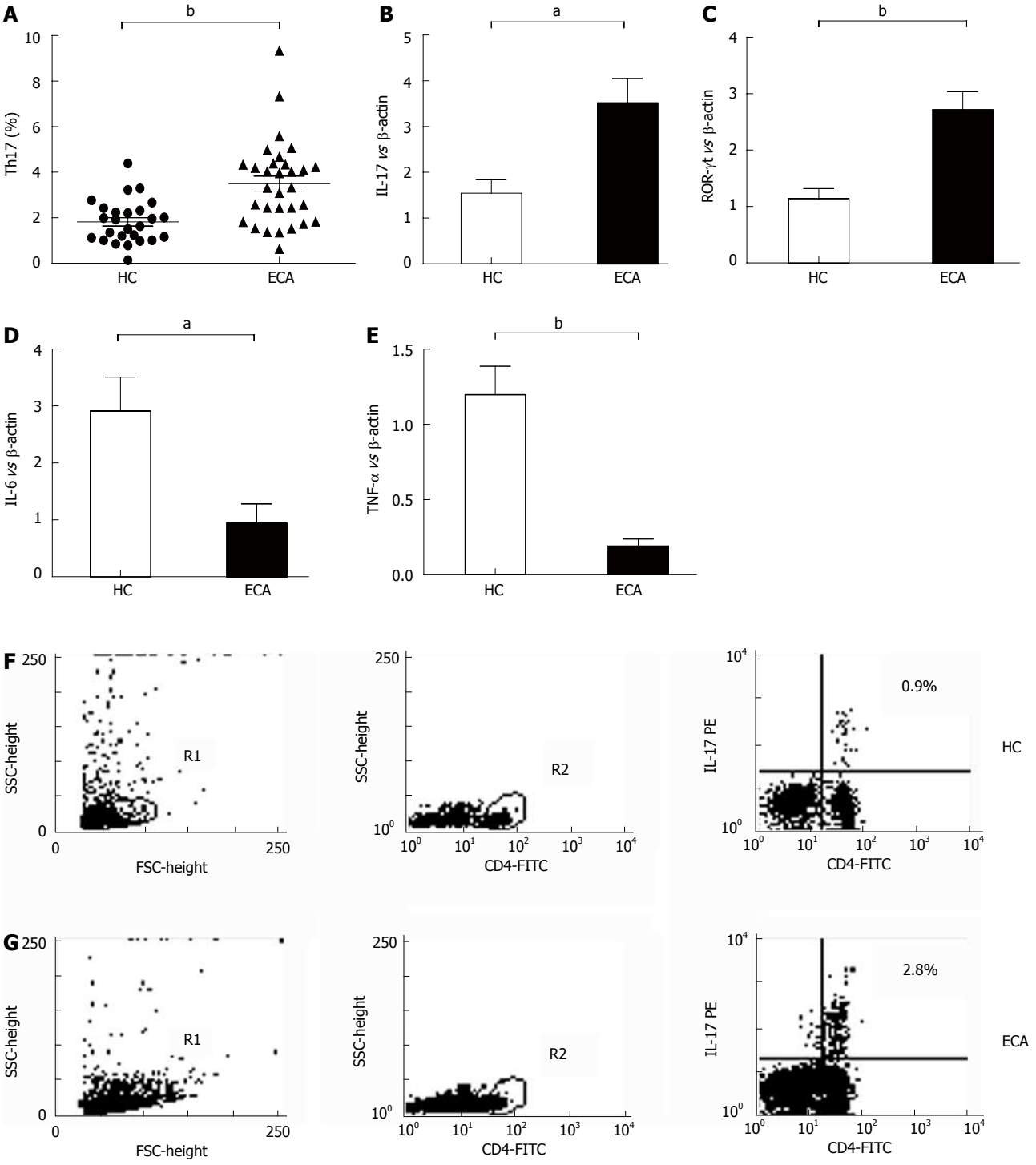


Figure 2 Levels of Th17 cells and Th17-related cytokines and transcriptional factor in the peripheral blood of esophageal cancer compared with healthy controls. A: Flow cytometric analysis of Th17 cells in the peripheral blood from ECA patients and healthy control (HC); B: The mRNA levels of IL-17 was determined by real-time PCR; C: The mRNA levels of ROR-γt was determined by real-time PCR; D: The mRNA levels of IL-6 was determined by real-time PCR; E: The mRNA levels of TNF-α was determined by real-time PCR; F: Representative flow diagrams of a healthy volunteer with marked elevation of circulating Th17 cells; G: Representative flow diagrams of an ECA patient with marked elevation of circulating Th17 cells are shown. Data were analyzed by the Student's *t*-test. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* HC group. ECA: Esophageal cancer; TNF-α: Tumor necrosis factor-α; IL: Interleukin; PCR: Polymerase chain reaction; FITC: Fluorescein isothiocyanate; FSC: Forward scatter; SSC: Side scatter.

Statistical analysis

Statistical comparisons between groups used the appropriate Student's *t*-test. Statistically significant correlation between two continuous variables was analyzed by the Spearman test. A *P*-value < 0.05 was considered significant. Calculations were performed using GraphPad Prism,

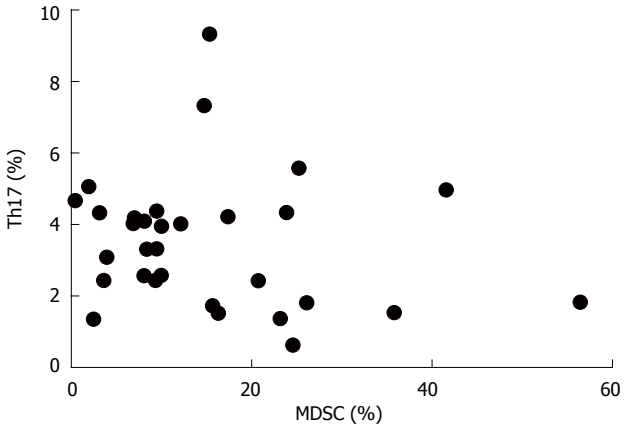


Figure 3 Correlation between the frequencies of myeloid-derived suppressor cells and Th17 cells in the peripheral blood of esophageal cancer patients. Data were analyzed by the Spearman test. MDSCs: Myeloid-derived suppressor cells.

Version 5.0, software (San Diego, CA, United States).

RESULTS

Increased frequencies of myeloid-derived suppressor cells in the peripheral blood of esophageal cancer patients

In the present study, MDSCs was defined as HLA-DR$^-$/CD14$^-$/CD11b$^+$/CD33$^+$ cells. The frequencies of MDSCs were determined by multicolor flow cytometry and calculated as the percent (%) of total nucleated cells in whole blood samples. As shown in Figure 1A, D and E, there was an increased prevalence of MDSCs in the peripheral blood from ECA patients (15.21% ± 2.25%) when compared with healthy control (HC) (1.10% ± 0.12%, $P < 0.0001$).

Increased plasma arginase 1 and inducible NO synthase mRNA expression in the peripheral blood from esophageal cancer patients

To investigate whether the products of MDSCs were also elevated in ECA patients, we determined the plasma levels of Arg1 by ELISA and the mRNA levels of iNOS by real-time PCR. As shown in Figure 1B, the plasma levels of Arg1 in ECA patients were significantly higher than those in HC (28.28 ± 4.10 ng/mL *vs* 9.57 ± 1.51 ng/mL, $P = 0.0003$). iNOS mRNA levels in the peripheral blood of ECA patients also showed a threefold increase compared with HC ($P = 0.0162$, Figure 1C).

Increased frequency of Th17 cells in the peripheral blood mononuclear cells of esophageal cancer patients

The frequencies of Th17 cells in PBMCs of ECA patients and HC were also determined by flow cytometry. As shown in Figure 2A, C and D, the frequencies of Th17 cells (CD4$^+$IL-17A$^+$) were significantly elevated in ECA patients when compared with HC (3.50% ± 0.33% *vs* 1.82% ± 0.19%, $P = 0.0001$).

mRNA expression analyses in peripheral blood mononuclear cells from esophageal cancer patients showed increased interleukin-17 and ROR-γt and decreased interleukin-6 and tumor necrosis factor-α

We also determined the mRNA levels of the Th17-related molecules IL-17, ROR-γt, IL-6 and TNF-α by real-time PCR. As shown in Figure 2B, increased mRNA expression of IL-17 and ROR-γt was observed in ECA patients compared with HC ($P = 0.0041$ and $P = 0.0004$, respectively), while the mRNA expression of IL-6 and TNF-α showed significant decreases ($P = 0.0049$ and $P < 0.0001$, respectively).

Correlation between myeloid-derived suppressor cells and Th17 cells in the peripheral blood from esophageal cancer patients and HC

We next analyzed the possible correlation between the frequencies of MDSCs and Th17 cells in the peripheral blood from ECA patients and HC. As shown in Figure 3, no obvious difference was found between the two groups ($r = -0.1725$, $P = 0.3534$).

Correlation of arginase 1, inducible NO synthase and Th17 cell-related molecules in the peripheral blood from HC and esophageal cancer patients

We examined the possible correlations between the mRNA levels of Arg1, iNOS and Th17-related molecules (IL-17, ROR-γt, IL-6 and TNF-α) in ECA patients. As shown in Figure 4D and E, Arg1 mRNA levels were positively correlated with levels of IL-6 ($r = 0.6404$, $P = 0.0031$) and TNF-α ($r = 0.7646$, $P = 0.0001$). Similarly, as shown in Figure 4I and J, iNOS mRNA levels were also positively correlated with levels of IL-6 ($r = 0.6782$, $P = 0.0007$) and TNF-α ($r = 0.7633$, $P < 0.0001$). However, no obvious correlations were observed with Arg1 or iNOS with IL-17 or ROR-γt (Figure 4A-C and 4F-G).

DISCUSSION

MDSCs are a heterogeneous cell population that was recently identified as a pivotal factor in the immunosuppressive network described in cancer, autoimmune disease, sepsis, infectious disease and trauma[1,15,16]. In cancer, MDSCs are responsible for T cell defects as well as angiogenesis and tumor cell motility. In this study, we evaluated the frequencies of MDSCs in the peripheral blood in ECA patients. Our study confirms previous findings about the significant increase of circulating MDSCs in a variety of cancers. An early study reported the accumulation of immature cells, including early stage myeloid cells and immature monocytes and DCs, in the blood of patients with head and neck, breast, and lung cancer[4]. Myeloid cells with immunosuppressive properties can also be found among monocytes and neutrophils circulating in the peripheral blood of patients with colon cancer and melanoma[5]. Patients with glioblastoma have increased MDSC counts (CD33$^+$HLADR$^-$) in their blood that are composed of neutrophilic (CD15$^+$, > 60%), lineage-

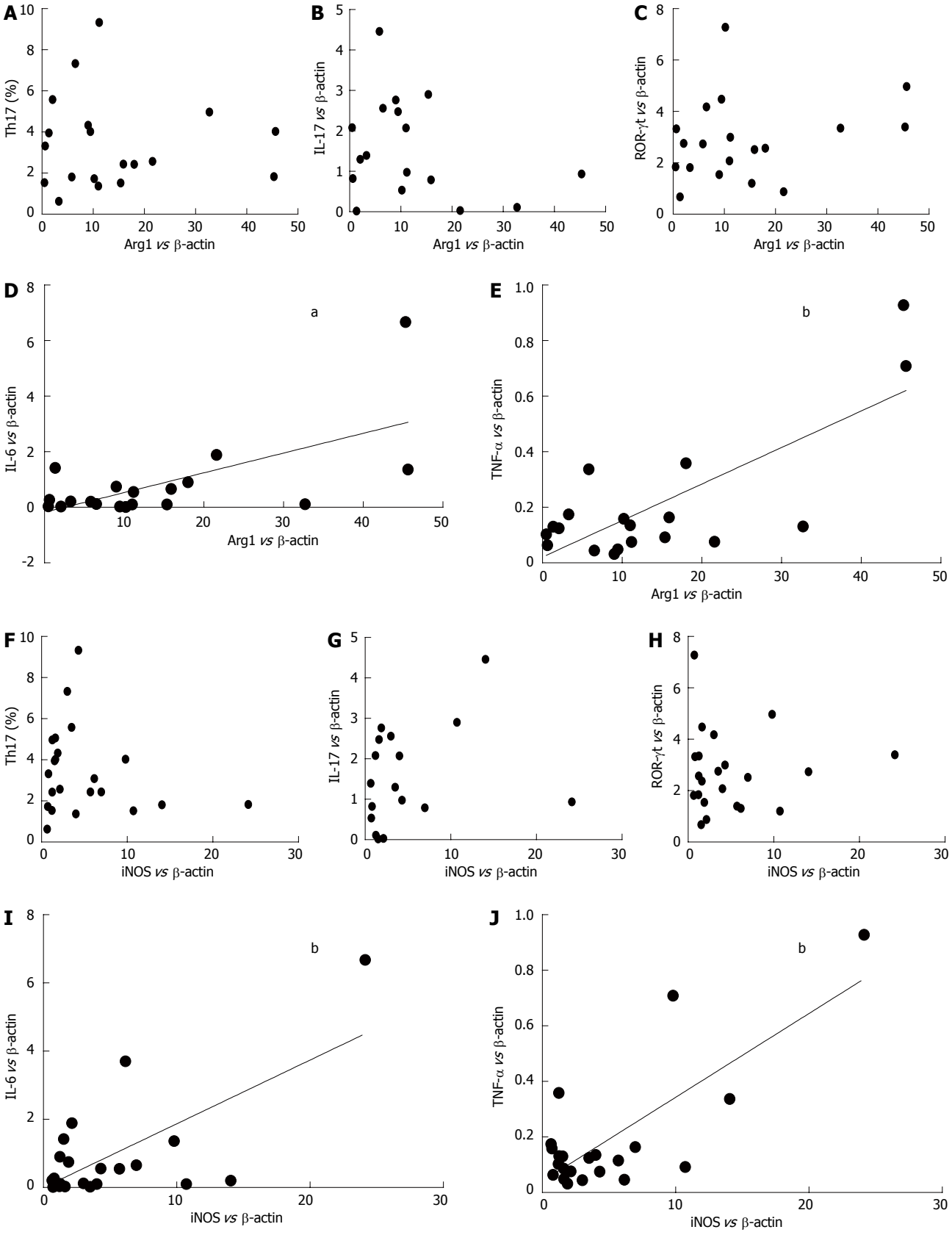


Figure 4 Correlations between the mRNA level of arginase 1 or inducible NO synthase and Th17-related molecules (interleukin-17, ROR-γt, interleukin-6 and tumor necrosis factor-α) in the peripheral blood of esophageal cancer patients. Data were analyzed by the Spearman test. [a]$P < 0.05$, [b]$P < 0.01$ *vs* β-actin. TNF-α: Tumor necrosis factor-α; IL: Interleukin; iNOS: Inducible NO synthase; Arg1: Arginase 1.

negative ($CD15^-CD14^-$, 31%), and monocytic ($CD14^+$, 6%) subsets[2]. It has also been reported that there was an accumulation and persistence of long-lived immature granulocytic MDSC with T cell-suppressive function and impaired migratory properties in the peripheral blood of squamous cell carcinoma of the head and neck (HNSCC), lung cancer and cancers of bladder and ureter[3]. Overall, these studies demonstrated the increase of circulating MDSCs in cancers, although the levels of MDSCs in peripheral blood varied across studies. These varying levels may partly be the result of differences in the type of cancers. Another explanation for these discrepancies may be methodological differences in which different markers and cell preparations were used to identify MDSCs.

In human, MDSCs are most prevalently considered as cells expressing CD11b but lacking the expression of CD14 or more narrowly defined as $HLA\text{-}DR^-CD33^+$ cells[4,17]. In this study, we used $CD14^-HLA\text{-}DR^-CD33^+CD11b^+$ as specific markers for human MDSCs in lysed whole blood. A recent study also reported elevated MDSCs in pancreatic, esophageal and gastric cancer and demonstrated that these cells were an independent prognostic factor and associated with the significant elevation of the Th2 cytokine interleukin-13[6]. The authors of such study focused on circulating MDSCs defined as $HLADR^-Lin1^{low/-}CD33^+CD11b^+$ and used a cell preparation of PBMCs rather than lysed whole blood, as was used in our study. However, both studies demonstrated elevations of plasma Arg1, which is the functional product of MDSCs, in ECA patients, compared with the controls. In addition to Arg1, we also found that iNOS mRNA levels in the peripheral blood of ECA patients showed a threefold increase compared with HC ($P = 0.0162$).

It is generally believed that Th17 cells and their associated cytokines IL-6, TNF, IL-1β, IL-23 and TGF-β may play important roles in promoting the growth and survival of cancer. Consistent with other previous reports, our study demonstrated that ECA patients exhibited a remarkable increase in the frequency of circulated Th17 cells. Furthermore, the mRNA levels of Th17-related cytokines, IL-17 and ROR-γt, have also been shown to be significantly increased[18]. Deans *et al*[19]reported that proinflammatory cytokines IL-6 and TNF-α were significantly overexpressed both at the mRNA and protein levels in cancer specimens compared with the mucosa from controls. However, in contrast to Deans's study, we found that the mRNA levels of IL-6 and TNF-α were obviously decreased in ECA patients. One possible explanation for this discrepancy may result partly from the limited scale of ECA subjects and determination methods. Another explanation is that, as is the case for most cancers, changes in peripheral blood could reflect partly or rather poorly the changes in tumor microenvironment. Further investigation should be performed to confirm the above inferences.

Recent data demonstrated that *in vivo* transfer of G-MDSCs inhibited Th17 immune responses and ameliorated experimental autoimmune encephalomyelitis (EAE)[20]. *In vitro*, $CD14^+HLA\text{-}DR^+$ monocytes promote the generation of IL-17-secreting $RORc^+$ Th17 cells when cocultured with naive CD4+ T cells[8]. These results suggest that the interaction of MDSC with Th17 cells would help to explain the pathogenesis of autoimmune diseases and cancers. In fact, the initial question that motivated our study was to determine whether there would be a positive correlation between circulating MDSCs and Th17 cells in individual ECA patient. As expected, there were high correlations between the mRNA levels of Arg1 or iNOS and IL-6 or TNF-α, which represent the products of MDSCs and Th17 cells, respectively. Although both the frequencies of MDSCs and Th17 cells in ECA were much higher than those in healthy controls, there was surprisingly no obvious correlation between the frequency of circulation MDSCs and Th17 cells. Consistently, there were no significant correlations between Arg1 or iNOS and Th17 cells, IL-17 or ROR-γt. There is no clear explanation for such unexpected results. Further studies are necessary to explore whether the peripheral increase of these cells could be found in local cancer tissue and whether a correlation between MDSCs and Th17 cells exist.

In conclusion, we have demonstrated a significant increase in circulating MDSCs and Th17 cells in ECA patients. The frequency of MDSCs is not correlated with Th17 cells. Arg1 and iNOS, the products of MDSCs, are consistently not associated with Th17 cells, IL-17 or ROR-γt but are positively correlated with Th17-related cytokines, including IL-6 and TNF-α. These results may bring new lines of investigation on the role of MDSCs and Th17 cells in ECA, possibly leading to new immunotherapy approaches based on the regulation of metabolites and cytokines.

COMMENTS

Background

Esophageal cancer (ECA) occurs when the tissue that lines the esophagus becomes malignant. Cancer of the esophagus is the sixth leading cause of cancer mortality worldwide, constituting approximately 2% of all malignant tumors. The precise mechanisms of initiation and progression of this disease are unclear. Recently, a suppressor cell population of myeloid lineage was identified, named myeloid-derived suppressor cells (MDSCs), which is capable of reducing antitumor as well as inflammatory immune responses. However, the characterization of MDSCs and their correlation with the newly identified pro-inflammatory Th17 cells in esophageal cancer remains unclear.

Research frontiers

MDSCs have gained much attention in recent years, mainly in the tumor immunology community. Because MDSCs are still a very poorly defined cell population, it will be difficult to specifically target these cells in cancer patients with the aim of engaging tumor-specific immune responses. The current research priority is how to identify better markers and the interaction of MDSCs with other cell types in different clinical settings, including in esophageal cancer.

Innovations and breakthroughs

Although the distribution of MDSCs and Th17 cells in esophageal cancer has been previously reported, a comprehensive correlation between circulating MDSCs and Th17 cells in ECA remains unclear. The present study was designed to evaluate the relationship between circulating MDSCs and Th17 cells by examining the cell frequencies and related cytokines and other products in the peripheral blood from patients with ECA. The authors confirmed previous findings about significant increases in circulating MDSCs and Th17 cells in ECA patients. However, the frequency of MDSCs is not correlated with Th17 cells.

Arginase I (Arg1) and inducible NO synthase (iNOS), the products of MDSCs, are consistently not associated with Th17 cells, IL-17 or ROR-γt but are positively correlated with Th17-related cytokines, including interleukin (IL)-6 and tumor necrosis factor-α (TNF-α). The results reveal the association between circulating MDSCs and Th17 cells, which may lead to new immunotherapy approaches for ECA based on the regulation of metabolites and cytokines.

Applications

Although it is difficult to specifically target these cells for immunotherapy, the results suggest that the regulation of metabolites and cytokines would be a potential therapeutic approach for esophageal cancer.

Terminology

MDSCs: Myeloid-derived suppressor cells (MDSCs), a heterogeneous population of cells that consists of immature myeloid cells, immature granulocytes, monocytes-macrophages, dendritic cells and myeloid progenitor cells, is capable of reducing anti-tumor as well as inflammatory immune responses; Th17 cells: a new member of the $CD4^+$T cell family that have been characterized as preferential producers of interleukin-17A (IL-17A), IL-17F, IL-21, IL-22, and TNF-α and have been found in increased numbers in tumor-bearing hosts.

Peer review

This paper reported on the relationship between MDSCs and Th17 cells in ECA patients. The presented data are preliminary but novel.

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BRIEF ARTICLE

Effect of the ginsenoside Rb1 on the spontaneous contraction of intestinal smooth muscle in mice

Lei Xu, Sui-Ping Huang

Lei Xu, Scientific Research Station for Post-doctoral Studies, Post-doctoral Research Centre, Zhongshan Affiliated Hospital, Guangzhou University of Traditional Chinese Medicine, Zhongshan 528400, Guangdong Province, China
Sui-Ping Huang, Department of Gastroenterology, Guangdong Provincial Traditional Chinese Medicine Hospital, Ersha Island Branch, Guangzhou 510405, Guangdong Province, China
Author contributions: Xu L performed the experiment and wrote the paper; Huang SP designed the research and analysed the data.
Supported by National Natural Science Foundation of China, No. 30873328; The State Administration of Traditional Chinese Medicine of the People's Republic of China, No. 06-075930
Correspondence to: Dr. Sui-Ping Huang, Department of Gastroenterology, Guangdong Provincial Traditional Chinese Medicine Hospital, Ersha Island Branch, Guangzhou 510405, Guangdong Province, China. doctorhsp@medmail.com.cn
Telephone: +86-20-87351238 Fax: +86-760-87602121
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Abstract

AIM: To investigate the effect and the possible mechanism of ginsenoside Rb1 on small intestinal smooth muscle motility in mice.

METHODS: Intestinal smooth muscle strips were isolated from male ICR mice (5 wk old), and the effect of ginsenoside Rb1 on spontaneous contraction was recorded with an electrophysiolograph. The effect of ginsenoside Rb1 on ion channel currents, including the voltage-gated K^+ channel current (IK_V), calcium-activated potassium channel currents (IK_{Ca}), spontaneous transient outward currents and ATP-sensitive potassium channel current (IK_{ATP}), was recorded on freshly isolated single cells using the whole-cell patch clamp technique.

RESULTS: Ginsenoside Rb1 dose-dependently inhibited the spontaneous contraction of intestinal smooth muscle by 21.15% ± 3.31%, 42.03% ± 8.23% and 67.23% ± 5.63% at concentrations of 25 μmol/L, 50 μmol/L and 100 μmol/L, respectively (n = 5, P < 0.05). The inhibitory effect of ginsenoside Rb1 on spontaneous contraction was significantly but incompletely blocked by 10 mmol/L tetraethylammonium or 0.5 mmol/L 4-aminopyridine, respectively (n = 5, P < 0.05). However, the inhibitory effect of ginsenoside Rb1 on spontaneous contraction was not affected by 10 μmol/L glibenclamide or 0.4 μmol/L tetrodotoxin. At the cell level, ginsenoside Rb1 increased outward potassium currents, and IK_V was enhanced from 1137.71 ± 171.62 pA to 1449.73 ± 162.39 pA by 50 μmol/L Rb1 at +60 mV (n = 6, P < 0.05). Ginsenoside Rb1 increased IK_{Ca} and enhanced the amplitudes of spontaneous transient outward currents from 582.77 ± 179.09 mV to 788.12 ± 278.34 mV (n = 5, P < 0.05). However, ginsenoside Rb1 (50 μmol/L) had no significant effect on IK_{ATP} (n = 3, P < 0.05).

CONCLUSION: These results suggest that ginsenoside Rb1 has an inhibitory effect on the spontaneous contraction of mouse intestinal smooth muscle mediated by the activation of IK_V and IK_{Ca}, but the K_{ATP} channel was not involved in this effect.

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Key words: Ginsenoside Rb1; Intestinal smooth muscle; Intestinal smooth muscle cell; Potassium channel; Spontaneous contraction; Whole-cell patch clamp technique

Peer reviewers: Dr. Adrian Gerard Cummins, Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia; Zhao-Xiang Bian, Professor, School of Chinese Medicine, Hong Kong Baptist University, 1/F, Jockey Club School of Chinese Medicine Building, 7 Baptist University Road, Hong Kong, China

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INTRODUCTION

Sijunzi decoction (SJZD) is one of the most famous and widely used traditional prescriptions. This prescription contains four common herbs, including Panax ginseng, Poria cocos, Atractylodes macrocephala and Glycyrrhiza uralensis, and it has been used either alone to replenish or invigorate intestinal and stomach function or as a complement to other herbs during treatment of other diseases, such as poor health and cancer[1,2]. Ginseng, the root of Panax ginseng C. A. Meyer (Araliaceae), is a principal component of SJZD. Ginsenoside, a component of ginseng, has a four-ring steroid-like structure with attached sugar moieties. Recently, ginseng's chemical and pharmacological properties have been reported by many investigators[3,4]. Approximately 30 ginsenosides have been isolated and identified from the Panax ginseng root. These ginsenosides appear to be responsible for most of the pharmacological effects of ginseng. Many reports made it evident that ginseng saponins, or ginsenosides, have various effects on gastrointestinal motility. Ginsenosides modulate the pacemaker activities of the interstitial cells of Cajal (ICCs), making the ICCs targets for ginsenosides, and their interaction can affect intestinal motility[5]. The aqueous extract of Ginseng Radix possesses ameliorative properties and improves carbachol-induced accelerated small intestinal transit, and Rb1 contributed to the suppressive effects of Ginseng Radix on intestinal motility. Rb1 is one representative of the compounds contained in Ginseng Radix that is capable of ameliorating the accelerated transit of the small intestine[6]. However, the mechanism of Rb1 modulation of gastrointestinal motility has not been clearly demonstrated. Based on the studies cited above, it could be deduced that ICCs and gastrointestinal smooth muscle cells might be targets for Rb1. In this study, we attempted to determine the effect of ginsenoside Rb1 on the motility of intestinal smooth muscle and determine its mechanism.

MATERIALS AND METHODS

Preparation of intestinal smooth muscle and isometric measurement

Five-week-old male ICR mice (provided by the Experimental Animal Centre of the Chinese Academy of Sciences, Shanghai) weighing approximately 30 g were sacrificed by cervical dislocation. The small intestines were removed and kept in Krebs solution. After removing the mucosal and submucosal layers, single circular muscle bundles with the attached longitudinal muscle layer were prepared. Approximately 2 mm × 6 mm muscle strips were needed and were fixed in a vertical chamber (5-mL capacity containing 5 mL CO_2/bicarbonate-buffered Krebs solution bubbled with 5% CO_2/95% O_2). The chamber was maintained at 37 ℃ using a water jacket. One end of the chamber was attached to an isometric force transducer (RM6240C, Chengdu Instrument Factory, China) to record the contraction. The muscle strip was incubated at the appropriate tension[7].

Cell preparation and electrophysiological recording

Intestinal smooth muscle cells were freshly isolated from mice. The intestine was rapidly cut, and the mucosal layer was separated from the muscle layers in a Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS). The circular muscle layer was dissected from the longitudinal layer using fine scissors and was cut into small segments (2 mm × 3 mm). These segments were incubated in a medium modified from Kraft-Bruhe (K-B) solution for 30 min at 4 ℃. The segments were subsequently incubated for 10-12 min at 36 ℃ in Ca^{2+}-free PSS digestion medium containing collagenase (0.5 mg/mL, Worthington), DTT [0.5 mg/mL, Sigma Aldrich (St. Louis, MO, United States)], papain [1.5 mg/mL, Sigma Aldrich (St. Louis, MO, United States)] and bovine serum albumin (4 mg/mL, Biotech Grade)[7,8]. After digestion, the supernatant was discarded, and the softened muscle segments were transferred into the modified K-B solution. The single cells were dispersed by gentle trituration using a wide-bore fire-polished glass pipette. The isolated intestinal smooth muscle cells were incubated in a modified K-B solution at 4 ℃ until use on the same day. Several drops of the cell suspension were dropped into a perfusion bath, which was fixed on the stage of an inverted phase-contrast microscope for 15-20 min before the experiments. Next, the cells were perfused with PSS at a rate of 1-1.5 mL/min. A single 4-channel perfusion system (BPS-4, ALA, United States) was used to exchange the solution.

A conventional whole-cell patch clamp configuration was used to record the K_{ATP} channel current (IK_{ATP}), the spontaneous transient outward currents (STOC) and the voltage-gated K^+ channel current (IK_V). To record IK_{ATP}, the membrane potential was clamped at -60 mV. The pipette solution consisted of the following (mmol/L): KCl 107, KOH 33, Hepes 10, $MgCl_2$ 1, Na_2ATP 0.1, NaADP 0.1, and GTP 0.3, adjusted to a pH of 7.2 with NaOH. To observe the effect of Rb1 on IKv, we applied a depolarising step pulse to the cells, and the membrane potential was clamped at -60 mV. The pipettes were filled with solution containing the following (mmol/L): KCl 20, potassium-aspartic acid 110, di-tris-creatine phosphate 2.5, disodium creatine phosphate 2.5, MgATP 5, Hepes 5, $MgCl_2$ 1.0, and EGTA 10, adjusted to a pH of 7.3 with KOH. To record STOC, the holding potential was clamped at -20 mV. The pipettes were filled with a solution containing the following (mmol/L): KCl 140, $MgCl_2$ 5, K_2ATP 2.7, Na_2GTP 0.1, disodium salt 2.5, Hepes 5, and EGTA 0.1, adjusted to a pH of 7.2 with Tris. The patch pipettes were pulled from borosilicate glass capillaries using a pipette puller (PC-10, Narishige Group, Japan). The current was amplified with an EPC-10 patch

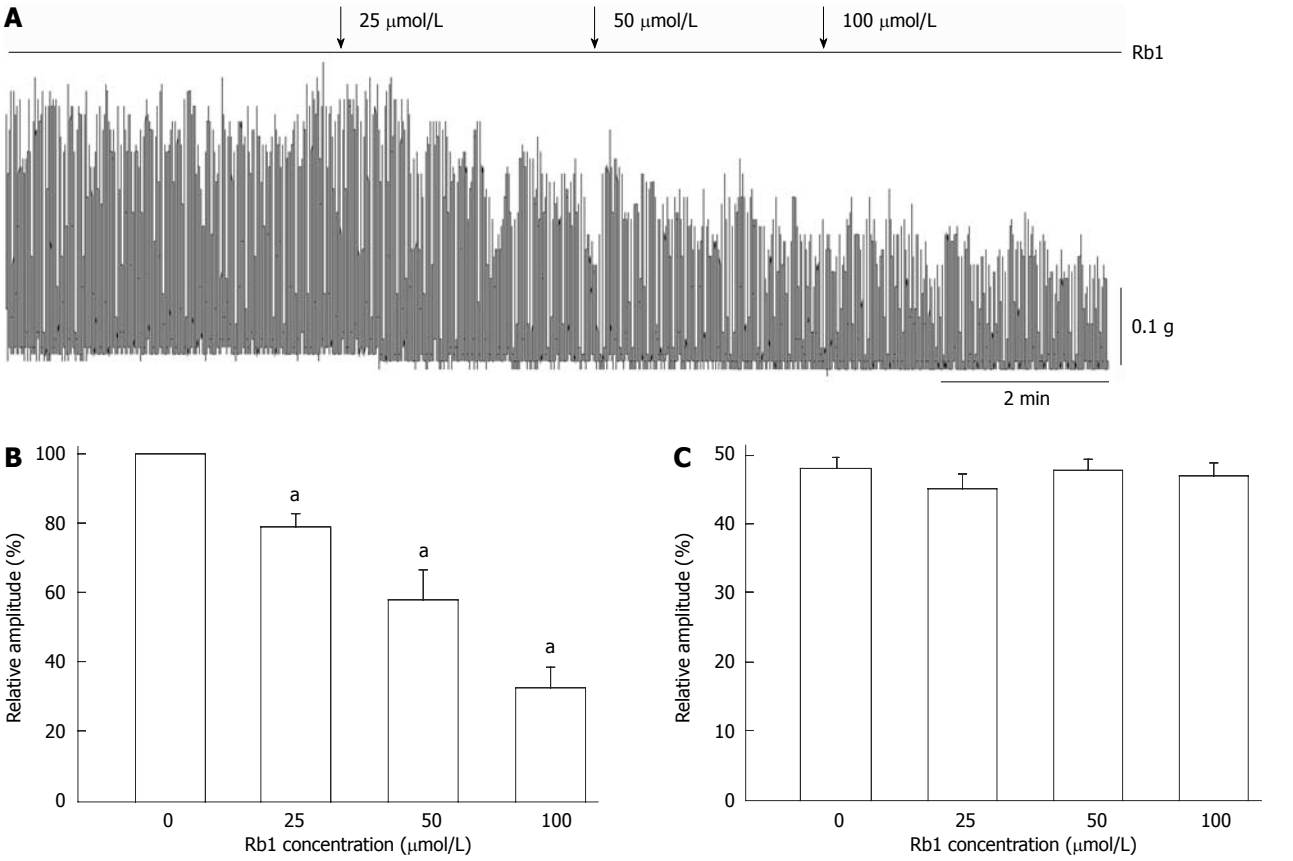


Figure 1 Effect of Rb1 on spontaneous contraction of intestinal smooth muscle. A: The representative effects of Rb1 on spontaneous contraction of intestinal smooth muscle in a dose-dependent manner; B, C: The amplitude of spontaneous contraction was decreased after Rb1 administration, but the frequency was unaffected. Values are expressed as means ± SE. $n = 5$, $^aP < 0.05$ *vs* control group.

clamp amplifier (HEKA Instruments, Germany) and digitised with a PCI-16 A/D converter (HEKA Instrument). All pipettes had a resistance of 3-5 MΩ[7,9,10].

All experimental protocols included in this manuscript were approved by the local animal care committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the Science and Technology Commission of the PRC (STCC Publication No. 2, revised 1988).

Solutions and drugs

Chemicals used included Ginsenoside Rb1 (purchased from Sichuan Weiqi Biological Technology CO., Ltd.), Glibenclamide [a K_{ATP} channel blocker, purchased from Tocris (Ellisville, Misso6yuri, United States)], tetraethylammonium (TEA, a non-selective potassium channel blocker), 4-aminopyridine (4-AP, a voltage-gated K^+ channel blocker), and tetrodotoxin (TTX, a blocker of voltage-dependent Na^+ channels) purchased from Sigma Aldrich (St. Louis, MO, United States). Ginsenoside Rb1 was dissolved first in dimethyl sulphoxide (DMSO) at a concentration of 200 mmol. For the intestinal smooth muscle isometric measurements, all chemicals were further diluted with Krebs solution to prepare the desired concentrations before use. In the electrophysiological recording experiment, Ginsenoside Rb1 was diluted with PSS to the final concentration immediately before use.

The ionic composition of the Krebs solution was as follows (in mmol/L): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 15.5, $H2PO_4^-$ 1.2, Cl^- 134, and glucose 11.5. The solution was aerated with O_2 containing 5% CO_2, and the pH was maintained at 7.2-7.3. The composition of Kraft-Bruhe (K-B) solution was as follows (in mmol/L): EGTA 0.5, Hepes 10, $MgCl_2$ 3, KCl 50, glucose 10, KH_2PO_4 20, Taurine 20, and L-Glutamic acid 50, adjusted to a pH of 7.4 with KOH. The composition of Ca^{2+}-free PSS was as follows (in mmol/L): NaCl 134.8, KCl 4.5, Hepes 10, $MgCl_2$ 1, and glucose 10, adjusted to a pH of 7.4 with Tris. The composition of PSS was as follows (in mmol/L): NaCl 134.8, KCl 4.5, Hepes 10, $MgCl_2$ 1, glucose 10, and $CaCl_2$ 2, adjusted to pH 7.4 with Tris. The pipette solution for recording the K_{ATP} channel current contained the following (mmol/L): KCl 107, KOH 33, Hepes 10, $MgCl_2$ 1, Na_2ATP 0.1, NaADP 0.1, and GTP 0.3, adjusted to a pH of 7.2 with NaOH. The pipettes were filled with a solution for IK_{Ca} containing the following (in mmol/L): KCl 140, $MgCl_2$ 5, K_2ATP 2.7, Na_2GTP 0.1, disodium salt 2.5, Hepes 5, and EGTA 0.1, adjusted to a pH of 7.2 with Tris. The pipettes were filled with solution for IK_V containing the following (in mmol/L): KCl 20, potassium-aspartic acid 110, di-tris-creatine phosphate 2.5, disodium-creatine phosphate 2.5, MgATP

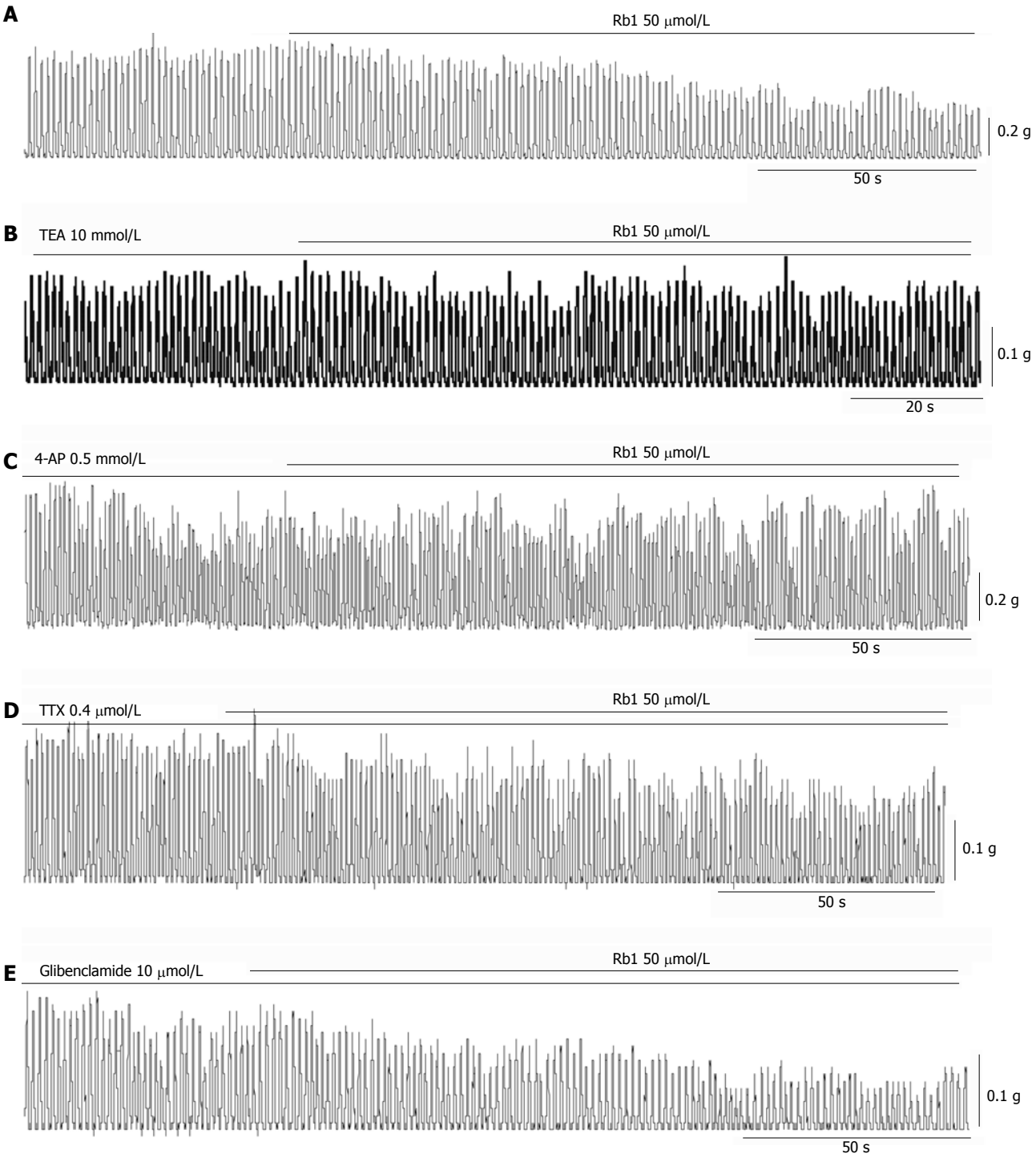


Figure 2 Effect of Rb1 on spontaneous contraction of the intestinal smooth muscle. A: Effect of Rb1 on spontaneous contraction of the intestinal smooth muscle; B-F: Effects of Rb1 (50 μmol/L) on spontaneous contraction of the intestinal smooth muscle pretreated with TEA (10 mmol/L), 4-AP (0.5 mmol/L), TTX (0.4 μmol/L) and Glibenclamide (10 μmol/L) respectively. TEA: Tetraethylammonium; 4-AP: 4-aminopyridine; TTX: Tetrodotoxin.

5, Hepes 5, $MgCl_2$ 1.0, and EGTA 10, adjusted to a pH of 7.3 with KOH.

Statistical analysis

Experimental values were expressed as the mean ± SD. Statistical significance was tested using Student's *t*-test, and probabilities of less than 5% ($P < 0.05$) were considered to be significant.

RESULTS

Effect of Rb1 on the spontaneous contraction of intestinal smooth muscle

In this study, Rb1 exhibited an inhibitory effect on the spontaneous contraction of intestinal smooth muscle strips in a dose-dependent manner (Figure 1). Rb1 suppressed spontaneous contraction by 21.15% ± 3.31%,

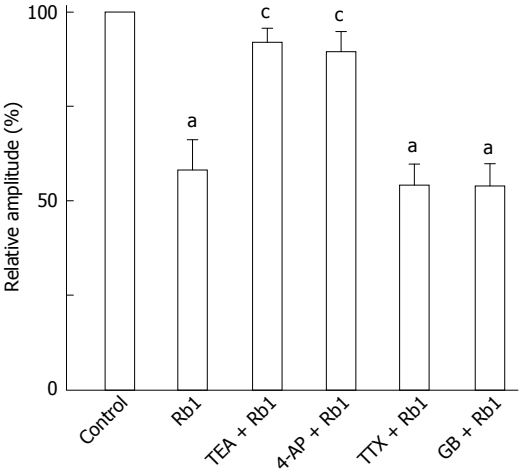


Figure 3 Summary in effect of Rb1on spontaneous contraction. Summary in effect of Rb1on spontaneous contraction of normal intestinal smooth muscles and those pretreated with TEA (10 mmol/L), 4-AP (0.5 mmol/L), Glibenclamide (10 μmol/L) and TTX (0.4 μmol/L) respectively. Values are expressed as means ± SE. $n = 5$, $^aP < 0.05$ *vs* control group; $^cP < 0.05$ TEA + Rb1 or 4-AP + Rb1 *vs* Rb1 group. TEA: Tetraethylammonium; 4-AP: 4-aminopyridine; TTX: Tetrodotoxin.

42.03% ± 8.23% and 67.23% ± 5.63% (Figure 1B, $n = 5$, $P < 0.05$) at concentrations of 25 μmol/L, 50 μmol/L and 100 μmol/L, respectively. Rb1-induced inhibition of spontaneous contraction appeared to decrease the amplitude of spontaneous contractions (Figure 1B), but the frequency was not changed (Figure 1C).

The Rb1-induced inhibitory effect on spontaneous contractions was almost completely abolished by 10 mmol/L TEA (a non-selective potassium channel blocker) and 0.5 mmol/L 4-AP (Figure 2B, C). The inhibitory percentage of Rb1 decreased from 42.03% ± 8.23% to 9.17% ± 3.54%, and the inhibitory percentage decreased from 10.90% ± 5.19% with TEA and 4-AP, respectively (Figure 3, $n = 5$, $P < 0.05$). After pre-treatment with 0.4 μmol/L TTX and 10 μmol/L glibenclamide, the inhibitory effect of Rb1 on spontaneous contraction was stable (Figure 2D, E). The inhibition percentages of Rb1 were 42.03% ± 8.23%, 46.12% ± 5.66% and 47.16% ± 3.99% in the control, TTX and glibenclamide groups, respectively (Figure 3, $n = 5$, $P > 0.05$).

Effect of Rb1 on voltage-gated K^+ channel current of intestinal smooth muscle cells

Previous experiments demonstrated that both TEA, a non-specific potassium channel blocker, and 4-AP, a specific delayed potassium channel blocker, significantly suppressed the inhibitory effect of RB1 on the spontaneous contraction of intestinal smooth muscle strips. These results indicate that Rb1-induced inhibition might be mediated by calcium-activated potassium channels and delayed repolarisation of the potassium channel. The effect of Rb1 on the IKv in intestinal smooth cells was observed in succession using the conventional whole-cell patch clamp technique. IKv was elicited by a step voltage command pulse from -40 mV to +100 mV at 20-mV increments for 400 ms at 10 s intervals. The membrane potential was clamped at -60 mV. Rb1 significantly increased IKv elicited by the step voltage command pulse (Figure 4A). Furthermore, based on the I-V relation curve, Rb1 increased IKv at all command potentials from +20 mV to +100 mV (Figure 4B). The IKv at +60 mV increased from 1137.71 ± 171.62 pA to 1449.73 ± 162.39 pA, which represented 132.11% ± 7.77% of the level in the control concentration (100%) of 50 μmol/L Rb1 (Figure 4C, $n = 6$, $P < 0.05$).

Effect of Rb1 on the Ca^{2+}-sensitive K^+ channel current of intestinal smooth muscle cells

IK_{Ca} is activated by intracellular Ca^{2+} and can be monitored by spontaneous transient outward currents (STOCs). STOCs are believed to represent the spontaneous, sporadic release of Ca^{2+} from storage sites in the cell in relation to Ca^{2+}-sensitive K^+ channels[8,11]. In this study, we observed that Rb1 (50 μmol/L) enhanced the amplitude of STOCs from 582.77 ± 179.09 mV to 788.12 ± 278.34 mV, which represented a 137.76% ± 11.95% increase from the control level (100%) (Figure 5A, C, $n = 5$, $P < 0.05$) without changing the frequency.

Effect of Rb1 on K_{ATP} channel current of intestinal smooth muscle cells

We investigated the effect of Rb1 on K_{ATP} channels using a whole-cell patch clamp. The inward current was activated at a holding potential of -60 mV following perfusion with a symmetrical 140 mmol/L K^+ solution (140 mmol/L KCl, 10 mmol/L glucose, 10 mmol/L Hepes, 1 mmol/L molgCl_2, and 0.1 mmol/L $CaCl_2$). Rb1 (50 μmol/L) did not change the K_{ATP} current (IK_{ATP}), which increased from 79.04 ± 35.88 pA to 81.32 ± 37.84 pA, representing a 102.29% ± 1.15% increase from the control level (100%) (Figure 5B, D, $n = 3$, $P < 0.05$).

DISCUSSION

Sijunzi decoction (SJZD) is widely used as a regular decoction in Chinese Traditional Medicine that can invigorate Pi viscera and replenish Qi. Conventionally, SJZD is useful for treating hypofunction of the spleen, a symptom that is partially equivalent to that of gastrointestinal motility disorders (e.g., abdominal distension and dyspepsia). The mechanism by which SJZD improves gastrointestinal disorder symptoms may relate to gastrointestinal hormones and motility. SJZD could correct deficiencies of the spleen and stomach, which are caused by digestive dysfunction to some extent[5]. Symptoms of rat models with Pi-deficiency could be significantly corrected to the normal level by SJZD treatment[4]. External nutrition plus SJZD treatment can improve and optimise cellular immune function and nutritional status in post-operative gastric cancer patients[12]. Recently, the major active components of SJZD, including ginsenoside, flavonoid, and triterpenoid, have been identified using LC/MS/MS[13]. Kim *et al*[14] reported that ginsenosides modulate the pace-

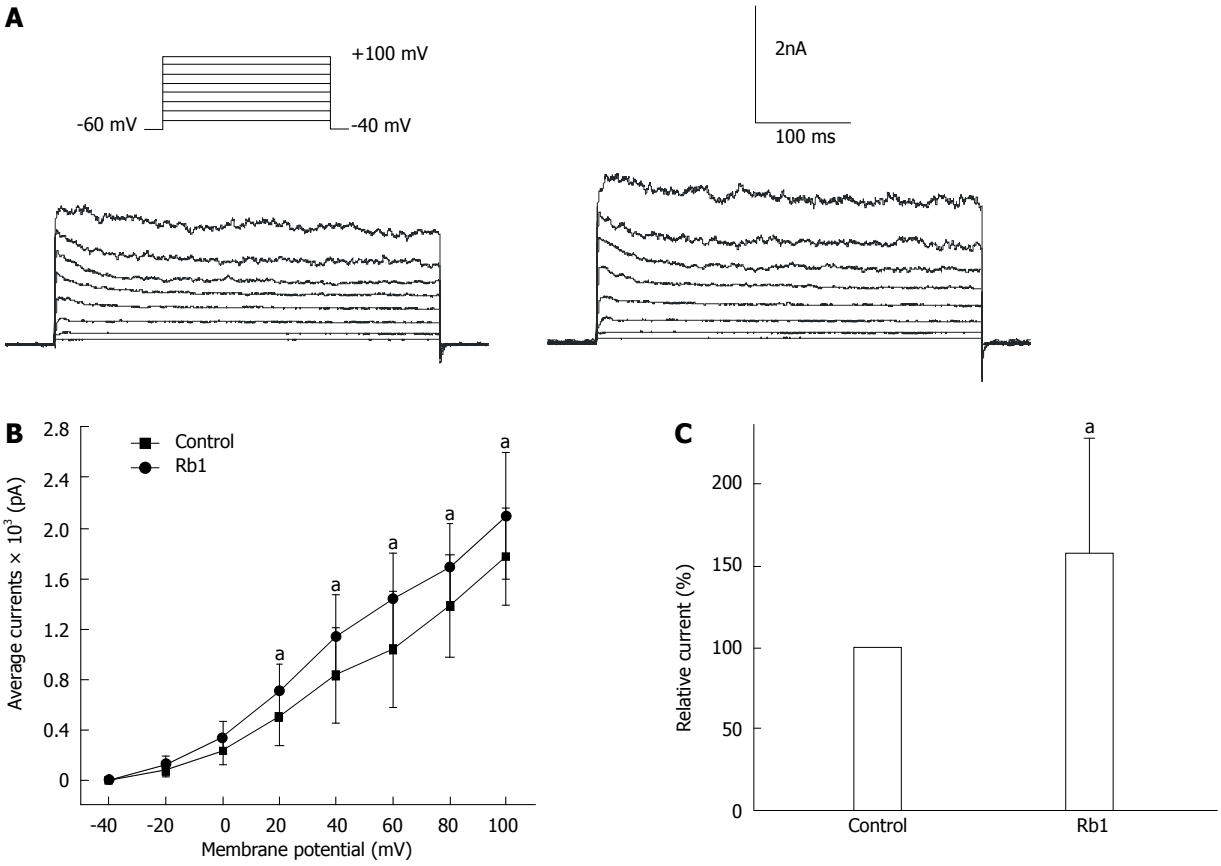


Figure 4 Effect of Rb1 on the voltage-gated K^+ channel current of intestinal smooth muscle cell in mouse. A: The raw trace; B: The I-V relation curve; C: Summary in effect of Rb1 (50 μmol/L) on the voltage-gated K^+ channel current at +60 mV. Values are expressed as means ± SE. $n = 6$, $^aP < 0.05$ *vs* control group.

maker activities of the ICCs. The ICCs can be targets for ginsenosides, and their interaction can affect intestinal motility. The ICCs and smooth muscle cells (SMCs) are coupled electrically, forming a multicellular syncytium. Activation of depolarising or hyperpolarizing ionic conductances in either cell type affects the total input resistance and excitability of the syncytium. For example, activation of K^+ channels in ICCs reduces excitability of coupled SMCs and reduces the likelihood of reaching the action potential threshold. Responses to other stimuli, such as hormones and paracrine substances, are likely to target both ICCs and SMCs, depending upon the expression of appropriate receptors and second-messenger pathways[15]. Hashimoto *et al*[16] reported that Rb1 was one representative of the compounds contained in Ginseng Radix that were capable of ameliorating the accelerated transit of the small intestine. To date, the mechanism of ginsenoside action on gastrointestinal (GI) smooth muscle has not been fully studied.

In this study, we found that ginsenoside Rb1 exerted an inhibitory effect on the spontaneous contraction of intestinal smooth muscles in mice by decreasing the amplitude of spontaneous contractions in a dose-dependent manner (Figure 1). The presence of TEA (10 mmol), a non-selective potassium channel blocker, partially blocked the inhibitory effect of Rb1 on spontaneous contraction (Figure 2B). This finding suggested that the inhibitory effect of ginsenoside Rb1 on the spontaneous contraction of intestinal smooth muscle in mice might be associated with K^+ channels; importantly, at least 20 species of potassium channel types are expressed by SMCs of the GI tract[15,17]. These species include voltage-gated K^+ channels, ATP-dependent K^+ channels, and Ca^{2+}-activated K^+ channels. We evaluated 4-AP, a voltage-gated K^+ channel blocker, which partially blocked the inhibitory effect of Rb1 on spontaneous contraction (Figure 2C). In contrast, glibenclamide, an ATP-dependent K^+ channel blocker, did not influence the inhibitory effect of Rb1 on spontaneous contraction (Figure 2E). In addition, the presence of TTX, a blocker of voltage-dependent Na^+ channels that can block enteric nerves, did not affect the inhibitory effect of Rb1 on spontaneous contraction (Figure 2D). Thus, the results indicated that the inhibitory effect of Rb1 on spontaneous contraction was associated with activation of K^+ channels in intestinal smooth muscle cells. A conventional whole-cell patch clamp configuration showed that Rb1 activated IK_V and IK_{Ca} (Figures 4, 5) without any influence on IK_{ATP} (Figure 5B, D). We concluded that Rb1 inhibited the spontaneous contraction of intestinal smooth muscles *via* increased Ca^{2+}-dependent K^+ channel currents and voltage-dependent K^+ channel currents. However, enteric nerves and K_{ATP} channels were not involved in this process. Next, to determine the Rb1-

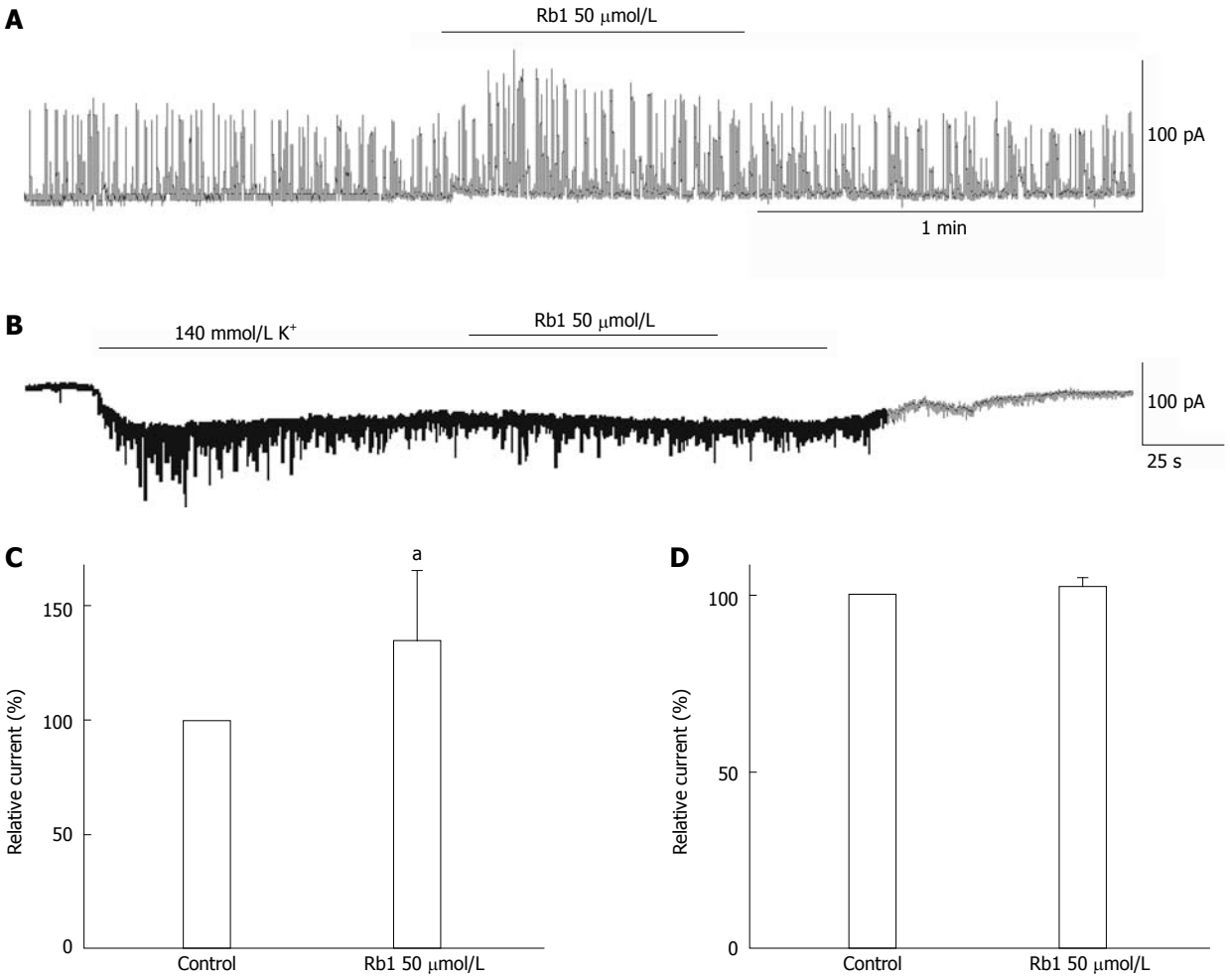


Figure 5 Effect of Rb1 on spontaneous transient outward currents and ATP sensitive potassium channel current of intestinal smooth muscle cell in mouse (*n* = 5). A: The raw trace of Rb1-induced effect on STOC of intestinal smooth muscle cell; B: The raw trace of Rb1-induced effect on IK_{ATP} of intestinal smooth muscle cell; C: Relative current evoked by Rb1 (50 μmol/L) on STOC, $n = 5$, $^aP < 0.05$ *vs* control group; D: Relative current evoked by Rb1 (50 μmol/L) on IK_{ATP} comparing with control group. Values are expressed as means ± SE, $n = 3$. IK_{ATP}: ATP sensitive potassium channel current; STOC: Spontaneous transient outward currents.

induced inhibitory effect on the spontaneous contraction of intestinal smooth muscle, the effect of Rb1 on the slow wave contraction of intestinal smooth muscle was observed. However, the amplitude and frequency of slow wave contraction was not affected by Rb1 (50 μmol/L, 100 μmol/L or 200 μmol/L, data not shown). The results indicated that the inhibitory effect of Rb1 on spontaneous contraction relies on the direct action of the compound with smooth muscle and not the ICCs themselves.

The broad ranges of resting potentials and electrical patterns of GI muscles are partly a function of the variable expression of K^+ channels in SMCs. At least 20 species of K^+ channels are expressed by SMCs in the GI tract[15]. The activation of potassium channels is the main determinant of cell membrane potential. Therefore, potassium channels participate in the regulation of smooth muscle tone. Activation of K^+ channels in the cell membrane allows K^+ efflux, causing a decrease in membrane potential and hyperpolarization. As a consequence, voltage-gated calcium channels in the cell membrane close, and the smooth muscle relaxes[18].

It has been reported that ginsenosides, including Rb1, regulate Ca^{2+} channels in chromaffin cells[19], sensory neurons[20] and ventricular myocytes[21]. Rb1 can alleviate cardiac hypertrophy *in vitro*, mediated by an inhibitive effect on elevated $[Ca^{2+}]i$[3]. The ginsenoside Rb1 suppressed ventricular myocyte shortening and intracellular Ca^{2+} in isolated cardiac myocytes[22]. These results indicate that the primary physiological or pharmacological targets of ginsenosides are Ca^{2+} channels. Li *et al*[23] reported that ginsenosides increased IK_{Ca} activity in endothelial cells. The modulation of IK_{Ca} activity stimulated by ginsenosides was inhibited by 0.5 mmol TEA but not by 0.5 mmol glibenclamide. In our study, we first discovered that potassium channels, especially the Ca^{2+}-dependent K^+ channels and voltage-dependent K^+ channels, were involved with the effects of Rb1 on the spontaneous contraction of intestinal smooth muscles in mice. This result is partially in accordance with the report of the action of ginsenosides by Li *et al*[23] and Kang *et al*[24].

In conclusion, ginsenoside Rb1 exerted an inhibitory effect on the spontaneous contraction of intestinal smooth muscles in mice by decreasing the amplitude of spontaneous contractions in a dose-dependent manner. The inhibitory effect of Rb1 is mediated by potentiating IKv and IK_{Ca} channel currents.

ACKNOWLEDGMENTS

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COMMENTS

Background

Gastrointestinal motility is a prominent research field of traditional Chinese medicine. Chinese herbal compounds, single Chinese erode drugs, Chinese herb extracts, and natural products have been experimentally investigated for their roles in promoting gastrointestinal motility.

Research frontiers

Ginsenosides, which are isolated from the root of Panax ginseng, appear to be responsible for most of the pharmacological effects of ginseng. Ginsenosides modulate the pacemaker activities of the interstitial cells of Cajal (ICCs), and the ICCs can be targets for ginsenosides, thereby affecting intestinal motility. Rb1 was one representative of the compounds contained in Ginseng Radix that was capable of ameliorating accelerated transit in the small intestine. Until now, the mechanism by which ginsenosides affect gastrointestinal smooth muscle had not been fully studied. This study focused on the mechanism by which ginsenosides affect gastrointestinal smooth muscle.

Innovations and breakthroughs

The results suggested that the ginsenoside Rb1 exerted an inhibitory effect on the spontaneous contraction of intestinal smooth muscle in mice in a dose-dependent manner. The inhibitory effect of Rb1 is mediated by current potentiation in the voltage-gated K^+ channel current (IK_V), calcium-activated potassium channel currents (IK_{Ca}) channels. This effect may be involved in the mechanism by which ginseng mediates gastrointestinal motility.

Applications

This study illustrates the mechanism by which Rb1 affects spontaneous contraction of intestinal smooth muscle in mice. These findings may clarify the pharmacological action of ginseng.

Peer review

This is a good descriptive study in which authors analyze the effect and the possible mechanism of ginsenoside Rb1 on small intestinal smooth muscle motility in mice. The results are interesting and suggest that ginsenoside Rb1 has an inhibitory effect on the spontaneous contraction of mouse intestinal smooth muscle mediated by the activation of IK_V and IK_{Ca}, but the ATP-sensitive potassium channel current channel was not involved in this effect.

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BRIEF ARTICLE

Tumor size as a prognostic factor in patients with advanced gastric cancer in the lower third of the stomach

Hong-Mei Wang, Chang-Ming Huang, Chao-Hui Zheng, Ping Li, Jian-Wei Xie, Jia-Bin Wang, Jian-Xian Lin, Jun Lu

Hong-Mei Wang, Chang-Ming Huang, Chao-Hui Zheng, Ping Li, Jian-Wei Xie, Jia-Bin Wang, Jian-Xian Lin, Jun Lu, Department of Gastric Surgery, Affiliated Union Hospital of Fujian Medical University, Fuzhou 350001, Fujian Province, China
Author contributions: Wang HM and Huang CM conceived of the study, analyzed the data, and drafted the manuscript; Zheng CH, Li P and Xie JW helped revise the manuscript critically for important intellectual content; Wang JB, Lin JX and Lu J helped collect data and design the study; and all authors read and approved the final manuscript.
Correspondence to: Chang-Ming Huang, Professor, Department of Gastric Surgery, Affiliated Union Hospital of Fujian Medical University, No. 29 Xinquan Road, Fuzhou 350001, Fujian Province, China. hcmlr2002@163.com
Telephone: +86-591-83363366 Fax: +86-591-83320319
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Abstract

AIM: To explore the impact of tumor size on outcomes in patients with advanced gastric cancer in the lower third of the stomach.

METHODS: We retrospectively analyzed the clinical records of 430 patients with advanced gastric cancer in the lower third of the stomach who underwent distal subtotal gastrectomy and D2 lymphadenectomy in our hospital from January 1998 to June 2004. Receiver-operating characteristic (ROC) curve analysis was used to determine the appropriate cutoff value for tumor size, which was measured as maximum tumor diameter. Based on this cutoff value, patients were divided into two groups: those with large-sized tumors (LSTs) and those with small-sized tumors (SSTs). The correlations between other clinicopathologic factors and tumor size were investigated, and the 5-year overall survival (OS) rate was compared between the two groups. Potential prognostic factors were evaluated by univariate Kaplan-Meier survival analysis and multivariate Cox's proportional hazard model analysis. The 5-year OS rates in the two groups were compared according to pT stage and pN stage.

RESULTS: The 5-year OS rate in the 430 patients with advanced gastric cancer in the lower third of the stomach was 53.7%. The mean ± SD tumor size was 4.9 ± 1.9 cm, and the median tumor size was 5.0 cm. ROC analysis indicated that the sensitivity and specificity results for the appropriate tumor size cutoff value of 4.8 cm were 80.0% and 68.2%, respectively (AUC = 0.795, 95%CI: 0.751-0.839, P = 0.000). Using this cutoff value, 222 patients (51.6%) had LSTs (tumor size ⩾ 4.8 cm) and 208 (48.4%) had SSTs (tumor size < 4.8 cm). Tumor size was significantly correlated with histological type (P = 0.039), Borrmann type (P = 0.000), depth of tumor invasion (P = 0.000), lymph node metastasis (P = 0.000), tumor-nodes metastasis stage (P = 0.000), mean number of metastatic lymph nodes (P = 0.000) and metastatic lymph node ratio (P = 0.000). Patients with LSTs had a significantly lower 5-year OS rate than those with SSTs (37.1% *vs* 63.3%, P = 0.000). Univariate analysis showed that depth of tumor invasion (χ^2 = 69.581, P = 0.000), lymph node metastasis (χ^2 = 138.815, P = 0.000), tumor size (χ^2 = 78.184, P = 0.000) and metastatic lymph node ratio (χ^2 = 139.034, P = 0.000) were significantly associated with 5-year OS rate. Multivariate analysis revealed that depth of tumor invasion (P = 0.000), lymph node metastasis (P = 0.019) and tumor size (P = 0.000) were independent prognostic factors. Gastric cancers were divided into 12 subgroups: pT2N0; pT2N1; pT2N2; pT2N3; pT3N0; pT3N1; pT3N2; pT3N3; pT4aN0; pT4aN1; pT4aN2; and pT4aN3. In patients with pT2-3N3 stage tumors and patients with pT4a stage tumors, 5-year OS rates were significantly lower for LSTs than for SSTs (P < 0.05 each), but there were no significant differences in the 5-year OS rates in LST and SST patients with pT2-3N0-2 stage tumors (P > 0.05).

CONCLUSION: Using a tumor size cutoff value of 4.8

cm, tumor size is a prognostic factor in patients with pN3 stage or pT4a stage advanced gastric cancer located in the lower third of the stomach.

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Key words: Gastric carcinoma; Prognosis; Receiver operating characteristic curve; The lower third of stomach; Tumor size

Peer reviewers: Mario M D'Elios, Professor, University of Florence, viale Morgagni 85, Florence, 50134, Italy; Dr. Wei-Dong Tong, MD, MS, BS, Associate Professor, Department of General Surgery, Veterans Affairs Medical Center, Medical College of Wisconsin, Milwaukee, WI 53295, United States

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INTRODUCTION

Gastric cancer is a common gastrointestinal malignancy in China and is the second most common cause of cancer-related deaths worldwide[1,2]. The identification of prognostic factors may be helpful in predicting and improving outcomes in patients with gastric cancer. Lymph node metastasis[3-5] and depth of tumor invasion[6-8] are the most important prognostic factors and are included in the Japanese Classification of Gastric Carcinoma (JCGC) and the American Joint Committee on Cancer tumor-nodes metastasis classification (AJCC TNM). Tumor size is another valuable clinicopathological feature because it can be measured easily before or during surgery and may be prognostic for survival in patients with gastric cancer[9-11]. Although tumor size is included in staging systems for lung and breast cancer, it has not been considered prognostic in gastric cancer. We therefore, retrospectively, analyzed the impact of tumor size on the prognosis of patients with advanced gastric cancer located in the lower third of the stomach.

MATERIALS AND METHODS

Patients

Patients undergoing curative resection (distal subtotal gastrectomy and D2 lymphadenectomy) for advanced gastric carcinoma (pT2-T4a stage) in the lower third of the stomach at the Department of Gastric Surgery, Affiliated Union Hospital of Fujian Medical University, Fuzhou, China, between January 1998 and June 2004 were included. Patients with gastric stump cancer, infiltration of surrounding organs (T4b) or distant metastases (hepatic, lung, peritoneal dissemination, or extraregional lymph nodes such as retropancreatic, mesenteric, and para-aortic lymph nodes) were excluded. After applying these criteria, 430 patients were included.

A surgical procedure was defined as curative if no grossly visible tumor tissue remained after the resection and the resection margins were histologically normal. Dissected lymph nodes were classified according to JCGC[12] criteria by specialist surgeons who reviewed the excised specimens after surgery. A total of 10 400 lymph nodes were dissected. The median number of dissected lymph nodes per patient was 24 (range, 6-61; mean 24.3 ± 8.8). Depth of tumor invasion, lymph node metastasis and tumor-nodes metastasis (TNM) stage were classified with respect to the seventh edition of AJCC TNM classification[13]. The metastatic lymph node ratio (MLR) was defined as the ratio of metastatic lymph nodes to the total number of dissected lymph nodes and categorized as MLR 0 (0%), MLR 1 (1%-9%), MLR 2 (10%-25%), MLR 3 (> 25%).

Routine follow-up consisted of physical examination, laboratory tests (including measurements of CEA, CA19-9 and CA125 concentrations), chest radiography, abdominopelvic ultrasonography and computed tomography (CT). Patients were followed-up every 3 mo during the first year, and every 6 mo or 12 mo thereafter, for a total of 5 years. Endoscopy was performed every 6 mo or 12 mo. All surviving patients were followed for more than five years. Survival was calculated from the date of diagnosis to last contact, date of death, or date when the survival information was collected. Of the 430 patients, 394 (93.0%) were followed-up.

Measurement of tumor diameter

In accordance with JCGC criteria[12], the resected stomach was opened along the greater curvature so that the whole mucosa could be observed. If the tumor was located on the greater curvature, the stomach was opened along the lesser curvature. The opened stomach was placed on a flat board with the mucosal side up and examined macroscopically. The lengths of the greater and lesser curvatures, as well as the attached portion of the esophagus and/or the duodenum and the size and thickness of the tumor, were recorded. Tumor size was measured as maximum tumor diameter (Figure 1). If tumor margins were unclear, the resected stomach was fixed in formalin for 1 h to make the margins clearer.

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 16.0 for Windows. The appropriate cutoff value for tumor size predicting 5-year survival was determined using the receiver-operating characteristic (ROC) curve, from which the area under the curve (AUC) was determined and the Youden index corresponding to each size was calculated. χ^2 tests were used to evaluate differences in proportions, and Student's *t*-tests were used to evaluate continuous variables. Five-year overall survival (OS) rates were calculated by the Kaplan-Meier method, with

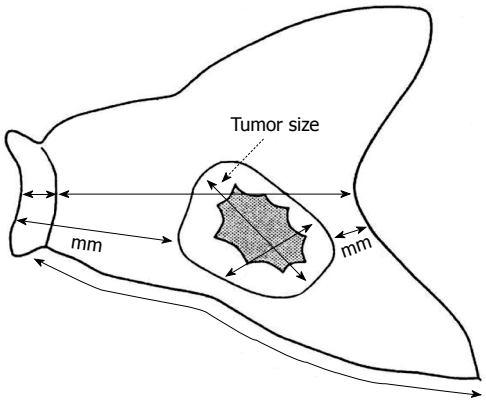


Figure 1 Resected stomach (mucosal side).

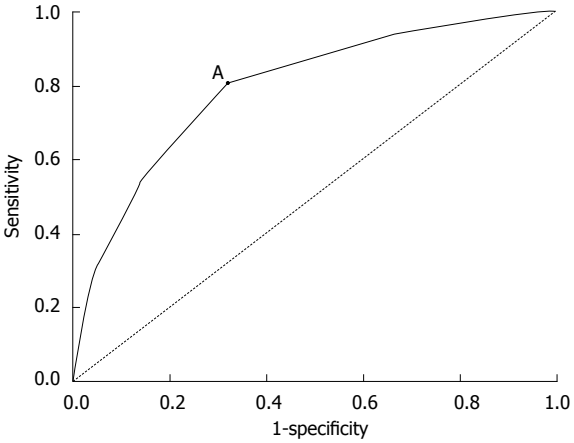


Figure 2 Receiver-operating characteristic curve. A: Shows sensitivity and specificity for a tumor size cutoff value of 4.8 cm were 80.0% and 68.2%, respectively (area under the curve = 0.795, *P* = 0.000).

groups compared by log-rank tests. Multivariate analysis was performed using a Cox's proportional hazard model. *P* values < 0.05 were considered statistically significant.

RESULTS

Clinicopathological characteristics of patients

We retrospectively analyzed a total of 430 patients with pT2-T4a gastric cancer located in the lower third of the stomach. Of these, 132 patients (30.7%) were women and 298 (69.3%) were men, with 187 patients (43.5%) ⩾ 60 years old. Postoperatively, 45 patients (10.5%) were classified as stage ⅠB, 49 (11.4%) as stage ⅡA, 78 (18.1%) as stage ⅡB, 65 (15.1%) as stage ⅢA, 82 (19.1%) as stage ⅢB and 111 (25.8%) as stage ⅢC. In addition, 117 patients (27.2%) were classified as stage pT2, 40 (9.3%) as pT3 and 273 (63.5%) as pT4a, while 105 patients (24.4%) were stage pN0, 92 (21.4%) were pN1, 94 (21.9%) were pN2, and 139 (32.3%) were pN3. Of the 430 patients, 325 (75.6%) had undifferentiated tumors. Based on the MLR classification, 70 patients (16.3%) were classified as MLR 0, 118 (27.4%) as MLR 1, 93 (21.6%) as MLR 2, and 149 (34.7%) as MLR 3.

Table 1 Correlation between other clinicopathologic characteristics and tumor size *n* (%)

Factors	LST	SST	*P*
Gender			0.221
Male	148 (66.7)	150 (72.1)	
Female	74 (33.3)	58 (27.9)	
Age (yr)			0.209
< 60	119 (53.6)	124 (59.6)	
⩾ 60	103 (46.4)	84 (40.4)	
Histological type			0.039
Differentiated	45 (20.3)	60 (28.8)	
Undifferentiated	177 (79.7)	148 (71.2)	
Borrmann type			0.000
Ⅰ / Ⅱ	50 (22.5)	111 (53.4)	
Ⅲ / Ⅳ	172 (77.5)	97 (46.6)	
Lymph node metastasis			0.000
pN0	35 (15.8)	70 (33.7)	
pN1	41 (18.5)	51 (24.5)	
pN2	52 (23.4)	42 (20.2)	
pN3	94 (42.3)	45 (21.6)	
Depth of invasion			0.000
pT2	28 (12.6)	89 (42.8)	
pT3	19 (8.6)	21 (10.1)	
pT4a	175 (78.8)	98 (47.1)	
TNM stage			0.000
ⅠB	9 (4.1)	36 (17.3)	
ⅡA	14 (6.3)	35 (16.8)	
ⅡB	33 (14.9)	45 (21.6)	
ⅢA	30 (13.5)	35 (16.8)	
ⅢB	54 (24.3)	28 (13.5)	
ⅢC	82 (36.9)	29 (14.0)	
Number of lymph nodes	8.02 ± 8.66	3.98 ± 5.65	0.000
MLR			0.000
0	35 (15.8)	70 (33.6)	
1	37 (16.7)	46 (22.1)	
2	41 (18.5)	43 (20.7)	
3	109 (49.0)	49 (23.6)	

LST: Large-sized tumor; SST: Small-sized tumor; MLR: Metastatic lymph node ratio; TNM: Tumor-nodes metastasis.

Cutoff value of tumor size

The mean ± SD tumor size was 4.9 ± 1.9 cm, and the median tumor size was 5.0 cm (range, 1.0-12.0 cm). ROC analysis indicated that a cutoff value of 4.8 cm yielded a sensitivity of 80.0% and a specificity of 68.2% in predicting survival after gastric surgery (AUC = 0.795, 95%CI: 0.751-0.839, *P* = 0.000) (Figure 2). Based on this cutoff value, the patients were divided into 2 groups, with 222 (51.6%) having large-sized tumors [large-sized tumors (LSTs), ⩾ 4.8 cm] and 208 (48.4%) having small-sized tumors [small-sized tumors (SSTs), < 4.8 cm].

Correlation analysis

When we analyzed the correlation between other clinicopathologic factors and tumor size (Table 1), we found that tumor size was significantly correlated with histological type (*P* = 0.039), Borrmann type (*P* = 0.000), depth of tumor invasion (*P* = 0.000), lymph node metastasis (*P* = 0.000), TNM stage (*P* = 0.000), mean number of metastatic lymph nodes (*P* = 0.000) and metastatic lymph node ratio (*P* = 0.000). SSTs were associated with dif-

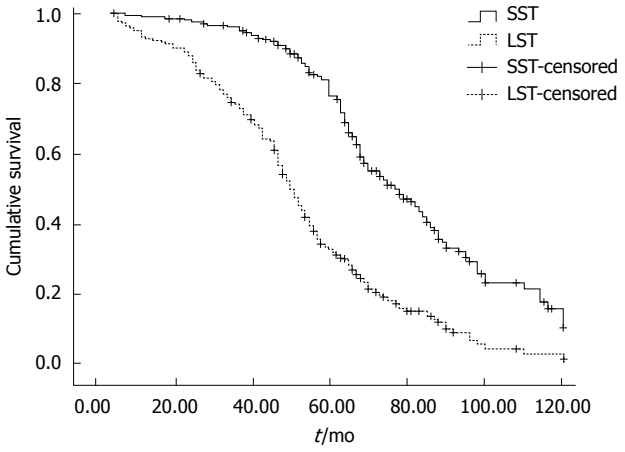


Figure 3 Overall survival curves of patients with large-sized tumor were significantly lower than those of patients with small-sized tumor. SST: Small-sized tumor; LST: Large-sized tumor.

ferentiated and Borrmann Ⅰ / Ⅱ types ($P < 0.05$ each), whereas LSTs were deeper and were associated with more extensive lymph node metastasis ($P < 0.05$). The mean number of metastatic lymph nodes was greater in patients with LSTs than with SSTs ($P < 0.05$).

Survival

The 5-year OS rate of all patients was 53.7%, being significantly lower in patients with LSTs than with SSTs (32.9% *vs* 76.7%; $\chi^2 = 78.184$, $P = 0.000$; Figure 3).

Univariate and multivariate survival analysis for all patients

Univariate analysis showed that depth of tumor invasion ($\chi^2 = 69.581$, $P = 0.000$), lymph node metastasis ($\chi^2 = 138.815$, $P = 0.000$), tumor size ($\chi^2 = 78.184$, $P = 0.000$) and metastatic lymph node ratio ($\chi^2 = 139.034$, $P = 0.000$) were significantly associated with 5-year OS rate, whereas patient age ($P = 0.613$), gender ($P = 0.240$) and histological type ($P = 0.361$) were not (Table 2). Multivariate analysis using a Cox's proportional hazards model revealed that depth of tumor invasion ($P = 0.000$), lymph node metastasis ($P = 0.019$) and tumor size ($P = 0.000$) independently predicted poor prognosis (Table 3).

Survival based on pT and pN stages in the LST and SST groups

Gastric cancers were divided into 12 subgroups: pT2N0; pT2N1; pT2N2; pT2N3; pT3N0; pT3N1; pT3N2; pT3N3; pT4aN0; pT4aN1; pT4aN2; and pT4aN3. The 5-year OS rates in these subgroups were compared in patients with LSTs and SSTs. We found that the 5-year survival rates of patients with pT2-3N3 stage and pT4a stage tumors were significantly lower in the LST than in the SST group ($P < 0.05$ each), but did not differ significantly in patients with pT2-3N0-2 stage tumors in the LST and SST groups ($P > 0.05$) (Table 4).

DISCUSSION

The prognostic value of tumor size in gastric cancer patients has recently received greater attention because tumor size can be measured easily before or during surgery; however, there is as yet no consensus formula to calculate the appropriate cutoff value for gastric tumor size. For example, tumor size of gastric cancer patients has been stratified into four subgroups (≤ 2 cm, ≤ 3 cm, ≤ 5 cm, and > 5 cm) by minimizing the estimated average expected distance (AED) objective function[14]. In another study of gastric cancer patients that used Cox's proportional hazards model to compare survival rates, a significant difference in survival was observed in patients with tumors < 10 cm and ≥ 10 cm[15]. We utilized ROC curve analysis to determine the appropriate tumor size cutoff value predicting 5-year OS rate in patients with advanced gastric cancer in the lower third of the stomach. In clinical and epidemiological fields, ROC analysis is frequently used to determine the cutoff values and reflect the correctness of a method of evaluation. According to the basic principle of ROC curves[16], when the Youden index is maximum, and the sensitivity is maximum, the corresponding tumor size is the appropriate cutoff value; besides, the method of evaluation can reflect predicted efficiency only when AUCs range between 0.7 and 0.9. From our ROC curves, a maximum sensitivity (0.800) at a tumor size cutoff value of 4.8 cm; our finding, of an AUC of 0.795 ($P = 0.000$), suggests that tumor size can reliably predict postoperative outcomes in patients with gastric cancer. Tumor size has shown positive associations with histological type, depth of tumor invasion, lymph node metastasis, peritoneal metastasis, blood vessel invasion and perineural inva-

Table 2 Univariate analysis of the patients' clinicopathological characteristics for overall survival

Factors	*n*	5-year OS (%)	χ^2	*P* value
Gender			1.655	0.198
Male	298	54.8		
Female	132	51.3		
Age (yr)			0.012	0.911
< 60	242	52.6		
≥ 60	188	55.1		
Tumor size (cm)			78.184	0.000
< 4.8	208	76.7		
≥ 4.8	222	32.9		
Depth of invasion			69.581	0.000
pT2	117	82.5		
pT3	40	56.5		
pT4a	273	41.2		
Histological type			0.835	0.361
Differentiated	105	66.5		
Undifferentiated	325	49.8		
Lymph node metastasis			138.815	0.000
pN0	105	85.9		
pN1	92	73.0		
pN2	94	40.4		
pN3	139	21.7		
MLR			139.034	0.000
0	70	85.9		
1	118	70.1		
2	93	54.1		
3	149	23.4		

OS: Overall 5-year survival rate; MLR: Metastatic lymph node ratio.

Table 3 Multiple stepwise regression analysis with the Cox proportional hazards model

Characteristics	B	SE	Wald	*P* value	RR	95%CI	
Depth of invasion			23.143	0.000			
pT3/pT2	0.604	0.228	7.032	0.008	1.829	1.171	2.857
pT4a/pT2	0.775	0.161	23.135	0.000	2.171	1.583	2.977
Lymph node metastasis			9.923	0.019			
pN1/pN0	0.578	0.280	4.268	0.039	1.783	1.030	3.085
pN2/pN0	1.130	0.420	7.224	0.007	3.095	1.358	7.055
pN3/pN0	1.601	0.528	9.204	0.002	4.959	1.763	13.954
MLR	0.061	0.170	0.130	0.719	1.063	0.762	1.483
Tumor size (cm)	0.762	0.123	38.524	0.000	2.143	1.684	2.726

MLR: Metastatic lymph node ratio; CI: Confidence interval; RR: Relative risk; B: Borrmann.

sion[17-20]. We found that LSTs were highly aggressive and malignant, with high disease stages. Compared with SSTs, LSTs showed deeper infiltration and were associated with more extensive lymph node metastasis, as well as having a significantly lower 5-year OS rate (37.1% *vs* 63.3%, $P < 0.05$), indicating that a cutoff of 4.8 cm could be used as a size criterion for gastric cancers.

The prognostic role of tumor size in gastric cancer remains unclear. An examination of 697 patients with gastric cancer who had undergone gastrectomy with curative intent found that tumor size was a predictor of survival in univariate analysis, but not in multivariate analysis[21]. In contrast, other researchers found that tumor size was an independent predictor of prognosis. For example, when patients were divided by tumor size into three subgroups, ⩽ 4 cm, ⩽ 10 cm, and > 10 cm in diameter, tumor size independently predicted patient survival[20]. Similarly, using a cutoff of 8 cm, tumor size was independently prognostic of survival[22]. We found that tumor size was significantly correlated with patient prognosis in both univariate and multivariate analysis, as were depth of tumor invasion and lymph node metastasis. Another study hypothesized that it was difficult to identify the most important variables associated with prognosis, and that the precise evaluation of the impact of tumor size on prognosis was feasible only when depth of invasion was specified[23]. Therefore, that study evaluated survival in patients with pT3 stage gastric cancer relative to pN stage in patients with LSTs and SSTs, finding that tumor size significantly influenced prognosis in pT3N2-3 stage tumors ($P = 0.004$). To eliminate depth of tumor invasion and lymph node metastasis as factors, we compared survival in patients in the LST and SST groups according to pT and pN stages. We observed no significant differences in 5-year OS of patients with pT2-3N0-2 stage tumors classified as LSTs and SSTs. In contrast, the 5-year OS rates were significantly lower in LST than in SST patients with pN3 stage or pT4a stage tumors ($P < 0.05$). In patients with pT2-3N0-2 stage tumors, the tumors likely did not infiltrate the serosa and had less extensive lymph node metastasis, reducing the likelihood of free cancer cells in the peritoneal cavity and decreasing the possibility of peritoneal recurrence. Tumor size, therefore, did not significantly affect postoperative survival in these patients. In patients with pN3 stage or pT4a stage LSTs, however, the interactions between tumors and lymphatic tissue were enhanced, thus increasing the likelihood of lymph node micrometastasis and diffusion to lymphatic vessels; the larger the area of the serosa invaded by tumor, the greater the likelihood for intraperitoneal dissemination, and the poorer the prognosis[24-26]. Tumor size was therefore correlated with survival of patients with pN3 stage or pT4a stage gastric cancer.

In conclusion, using a cutoff value of 4.8 cm, tumor size may be a prognostic factor in patients with pN3 stage or pT4a stage advanced gastric cancer located in the lower third of the stomach.

Table 4 Survival based on pT and pN stages in the large-sized tumor and small-sized tumor groups

	n (5-yr OS, %)		χ^2	*P* value
	LST	SST		
pT2				
pN0	9 (88.9)	36 (96.7)	0.260	0.610
pN1	11 (72.7)	26 (91.1)	0.000	0.986
pN2	4 (50.0)	16 (87.1)	0.066	0.797
pN3	4 (0.00)	11 (63.6)	7.661	0.006
pT3				
pN0	3 (66.7)	9 (100.0)	1.634	0.201
pN1	6 (66.7)	4 (75.0)	0.348	0.555
pN2	2 (0.0)	3 (66.7)	0.825	0.364
pN3	8 (12.5)	5 (40.0)	3.940	0.047
pT4a				
pN0	23 (68.7)	25 (83.1)	5.108	0.024
pN1	24 (54.2)	21 (75.4)	4.743	0.029
pN2	46 (27.2)	23 (61.9)	7.682	0.006
pN3	83 (6.9)	28 (48.6)	23.138	0.000

LST: Large-sized tumor; SST: Small-sized tumor; OS: Overall 5-year survival rate.

COMMENTS

Background

In addition to lymph node metastasis and depth of tumor invasion, tumor size is an important clinicopathological feature of gastric cancer because it can be measured easily before or during surgery. To date, however, the prognostic role of tumor size on survival in patients with gastric cancer remains unclear, with no consensus formula to calculate an appropriate cutoff value.

Research frontiers

Tumor size is included in the staging systems of many malignant diseases such as lung and breast cancer. Although tumor size has been reported to independently influence prognosis in patients with gastric cancer, an appropriate cutoff size has not been determined, especially for advanced gastric cancers located in the lower third of the stomach.

Innovations and breakthroughs

The authors utilized receiver-operating characteristic (ROC) curve analysis to determine the appropriate tumor size cutoff value and assessed the relationship between tumor size and overall survival rate.

Applications

The authors found that, using a cutoff of 4.8 cm, tumor size was a prognostic factor in patients with pN3 stage or pT4a stage advanced gastric cancers located in the lower third of the stomach. That is, patients with pN3 stage or pT4a stage tumors ⩾ 4.8 cm had a poorer prognosis than those with tumors < 4.8 cm.

Peer review

In assessing the effect of tumor size in a large number of patients (430) with advanced gastric cancer located in the lower third of the stomach, the authors

found that tumor size, using a cutoff of 4.8 cm, was prognostic in patients with pN3 stage or pT4a stage tumors. This finding has important clinical implications for gastrointestinal surgeons and for patient prognosis.

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CASE REPORT

Metachronous penile metastasis from rectal cancer after total pelvic exenteration

Yuta Kimura, Dai Shida, Keiichi Nasu, Hiroki Matsunaga, Masahiro Warabi, Satoru Inoue

Yuta Kimura, Dai Shida, Keiichi Nasu, Hiroki Matsunaga, Satoru Inoue, Department of Surgery, Tokyo Metropolitan Bokutoh Hospital, 4-23-15 Koto-bashi, Sumida-ku, Tokyo 1308575, Japan
Masahiro Warabi, Department of Pathology, Tokyo Metropolitan Bokutoh Hospital, 4-23-15 Koto-bashi, Sumida-ku, Tokyo 1308575, Japan
Author contributions: Kimura Y, Nasu K, Matsunaga H, Warabi M, and Inoue S collected the data, performed the treatment and wrote the paper; Shida D was responsible for writing the paper and its supervision.
Correspondence to: Dai Shida, MD, PhD, Department of Surgery, Tokyo Metropolitan Bokutoh Hospital, 4-23-15 Koto-bashi, Sumida-ku, Tokyo 1308575, Japan. dshida-tky@umin.ac.jp
Telephone: +81-3-36336151 Fax: +81-3-36336173
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Peer reviewers: Nadia Peparini, MD, PhD, Department of General Surgery "Francesco Durante", La Sapienza University, Viale del Policlinico,155, 00161 Rome, Italy; Joerg Trojan, MD, Professor, Medical Department 1, Johann Wolfgang Goethe-University Medical Center, Theodor-Stern-Kai 7, D-60590 Frankfurt, Germany

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Abstract

Despite its abundant vascularization and extensive circulatory communication with neighboring organs, metastases to the penis are a rare event. A 57-year-old male, who had undergone total pelvic exenteration for rectal cancer sixteen months earlier, demonstrated an abnormal uptake within his penis by positron emission tomography/computed tomography. A single elastic nodule of the middle penis shaft was noted deep within Bucks fascia. No other obvious recurrent site was noted except the penile lesion. Total penectomy was performed as a curative resection based on a diagnosis of isolated penile metastasis from rectal cancer. A histopathological examination revealed an increase of well differentiated adenocarcinoma in the corpus spongiosum consistent with his primary rectal tumor. The immunohistochemistry of the tumor cells demonstrated positive staining for cytokeratin 20 and negative staining for cytokeratin 7, which strongly supported a diagnosis of penile metastasis from the rectum. The patient is alive more than two years without any recurrence.

INTRODUCTION

The penis is an uncommon site of metastasis, and only around 300 such cases have been reported in the English-language literature[1,2]. A comprehensive review of the 305 reported cases showed the most frequent primary sites of metastatic penile tumors to be the bladder (34.7%), prostate (29.8%), rectum and sigmoid colon (15.7%), and kidney (6.5%)[1]. Approximately 50 cases of penile metastasis from either rectal or sigmoid colon cancer have been reported[1-3]. Ketata *et al*[4] reported the 20th case of penile metastasis from rectal cancer in 2007, and thereafter five other cases have been reported[5-9]. One-third of all penile metastases are synchronous, whereas the remaining two-thirds are detected a mean of 18 mo after discovery of the primary tumor[7]. About 90% of reported cases of penile metastasis are a part of widespread disease[2], thus isolated metastatic penile carcinomas are exceptionally rare. This report presents a case of metachronous isolated penile metastasis from rectal cancer after total pel-

vic exenteration, which was treated with a curative penile resection.

CASE REPORT

A 57-year-old male had a large rectal cancer which occupied the pelvis minor with massive invasion to both the bladder and prostate. He underwent sigmoid colostomy twenty three months previously. Nine courses of chemotherapy [oxaliplatin 100 mg intravenous (*iv*) on day 1, levofolinate 125 mg *iv* on day 1, 5-fluorouracil (5-FU) 500 mg *iv* bolus on day 1 and 5-FU 750 mg *iv* continuous infusion on days 1 and 2] had been administered after the first operation and the tumor slightly decreased in size without showing any distant metastasis. The patient underwent total pelvic exenteration, ileal conduit and pelvic reconstruction with a gracilis muscular flap sixteen months ago. There was no clinical or radiological evidence of distant metastasis at the time of resection. The histopathological examination revealed a moderately differentiated adenocarcinoma of the rectum invading to the bladder and prostate without lymph node metastasis (none out of 57). The disease was staged as T4 N0 M0 stage Ⅱ (Dukes Classification B). He made an uneventful recovery and was discharged on the nineteenth postoperative day.

No clinical or radiological evidence of recurrence was observed for nine months after total pelvic exenteration until he started complaining of intermittent bloody discharge from his penis. Positron emission tomography/computed tomography (PET/CT) showed high 18-F fluorodeoxyglucose uptake in the penis (Figure 1A). A physical examination at that time showed a single 4 cm elastic nodule of the penis shaft in the middle, deep within Bucks fascia, which had slight tenderness. Penile magnetic resonance imaging (MRI) showed a low intensity lesion on a gadolinium-enhanced fat-suppressed T1-weighted image (Figure 1B). Contrast enhanced computed tomography (CECT) revealed neither other distant metastases nor local recurrence, and the serum tumor marker carcinoembryonic antigen was within normal limits.

The patient underwent total penectomy based on the diagnosis of penile metastasis from rectal cancer with the resection of all the residual urethra, as a curative resection. The tumor was localized in the corpus spongiosum, and had almost completely replaced it (Figure 2). A histopathological examination of the resected specimen revealed an increase of well differentiated adenocarcinoma in the corpus spongiosum, consistent with his primary rectal tumor. Immunohistochemistry of tumor cells demonstrated positive staining for cytokeratin 20 and negative staining for cytokeratin 7 (Figure 3), which supported the diagnosis because the majority of rectal cancer does not stain for cytokeratin 7, but instead does stain for cytokeratin 20, and also because urothelial cells commonly shows positive staining for cytokeratin 7[4]. He underwent adjuvant chemotherapy, and now is good in health for more than two years since the last surgery with no recurrence.

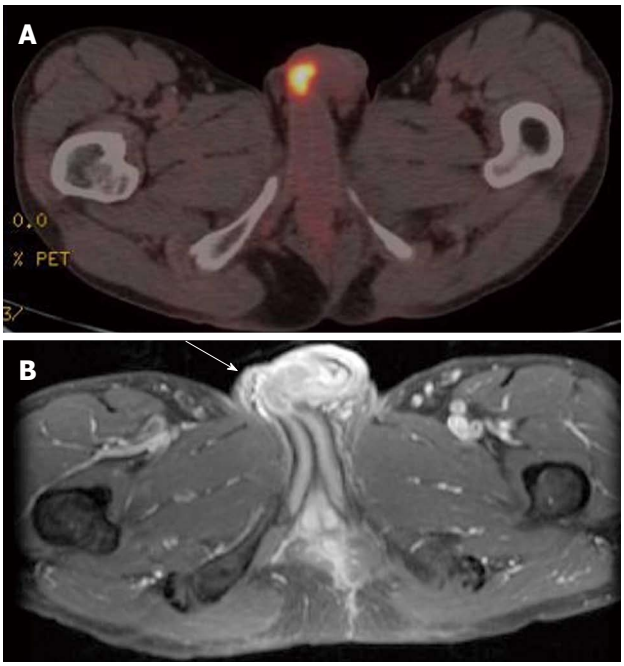


Figure 1 Positron emission tomography and magnetic resonance imaging image. A: Positron emission tomography/computed tomography showing high 18-F fluorodeoxyglucose uptake in the penis; B: Gadolinium-enhanced fat-suppressed T1-weighted image showing a low intensity lesion on the middle penis shaft (white arrow).



Figure 2 Resected specimen showing penile metastasis. A: Total penectomy with all the residual urethra; B: The tumor was localized in the corpus spongiosum, and had almost completely replaced it.

DISCUSSION

A metastatic penile tumor is rare despite the rich vascularity and the end arterial nature of its supply. Various mechanisms of penile metastasis have been suggested; retrograde venous spread, retrograde lymphatic spread,



Figure 3 Histopathological findings of the tumor. A histopathological examination revealing well differentiated adenocarcinomas in the corpus spongiosum (A) with negative staining for cytokeratin 7 (B) and positive staining for cytokeratin 20 (C) by immunostaining.

arterial spread, implantation and secondary to instrumentation or direct extension[1]. Among these mechanisms, retrograde venous spread from the pudendal venous system into the dorsal venous system of the penis appears to be the most common mode of spread[7]. Retrograde lymphatic spread into penile lymphatic channels after obstruction of inguinal and hypogastric nodes, is also considered[2]. One or both corpora cavernosa is the most common site of penile metastasis, and the glans penis and corpus spongiosum are rarely involved[1,10]. However, the current tumor was localized not in the corpora cavernosa but in the corpus spongiosum without widely disseminated disease, which seems to be extremely rare. These findings suggest that cancer cells in the residual urine at the time of total pelvic exenteration had likely become implanted in the corpus spongiosum.

Penile metastasis tends to show a poor prognosis because metastasis to the penis, in most cases, tends to be part of widely disseminated disease. The majority of patients die within one year[5]. Therefore, many treatment algorithms against penile metastases are described. The treatment is essentially palliative, and the combination of radiotherapy with chemotherapy produces only occasional responses. Some cases are treated by penectomy, an aggressive surgical excision, in order to palliate such symptoms as priapism and pain. Penectomy can offer the possibility of cure in patients with disease localized to the penis, like the current patient, even though such cases may be rare.

In conclusion, this report described a case of penile metastasis from rectal cancer. The patient underwent a complete cure resection with subsequent adjuvant chemotherapy, rather than palliative treatment. Penile involvement is usually a marker of widespread hematogenous dissemination which indicates a poor prognosis, but such a prognosis does not seem applicable in the current case.

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CASE REPORT

Primary hepatic leiomyosarcoma with liver metastasis of rectal cancer

Kiyoto Takehara, Hideki Aoki, Yuko Takehara, Rie Yamasaki, Kohji Tanakaya, Hitoshi Takeuchi

Kiyoto Takehara, Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan
Hideki Aoki, Yuko Takehara, Kohji Tanakaya, Hitoshi Takeuchi, Department of Surgery, National Hospital Organization Iwakuni Clinical Center, Yamaguchi 740-8510, Japan
Rie Yamasaki, Department of Clinical Examination, National Hospital Organization Iwakuni Clinical Center, Yamaguchi 740-8510, Japan
Author contributions: Takehara K performed the surgery and was involved in writing the manuscript; Aoki H instructed during surgery and reviewed the manuscript; Takehara Y assisted with the surgery and patient care; Yamasaki R carried out the histopathological examination; Tanakaya K and Takeuchi H supervised the report.
Correspondence to: Kiyoto Takehara, MD, Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan. t_kiyotosan@cyberbb.com
Telephone: +81-86-2237151 Fax: +81-86-2357636
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Abstract

Primary hepatic leiomyosarcoma is a particularly rare tumor with a poor prognosis. Curative resection is currently the only effective treatment, and the efficacy of chemotherapy is unclear. This represents the first case report of a patient with primary hepatic leiomyosarcoma co-existing with metastatic liver carcinoma. We present a 59-year-old man who was diagnosed preoperatively with rectal cancer with multiple liver metastases. He underwent a curative hepatectomy after a series of chemotherapy regimens with modified FOLFOX6 consisting of 5-fluorouracil, leucovorin and oxaliplatin plus bevacizumab, FOLFIRI consisting of 5-fluorouracil, leucovorin and irinotecan plus bevacizumab, and irinotecan plus cetuximab. One of the liver tumors showed a different response to chemotherapy and was diagnosed as a leiomyosarcoma following histopathological examination. This case suggests that irinotecan has the potential to inhibit the growth of hepatic leiomyosarcomas. The possibility of comorbid different histological types of tumors should be suspected when considering the treatment of multiple liver tumors.

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Key words: Leiomyosarcoma; Rectal cancer; Metastasis; Chemotherapy; Surgery

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INTRODUCTION

Hepatic sarcomas constitute only 1%-2% of primary malignant tumors of the liver, and primary hepatic leiomyosarcoma is particularly rare[1]. The prognosis of primary hepatic leiomyosarcoma is poor, but long-term survival is possible if complete resection can be achieved[2]. We herein report a case of primary hepatic leiomyosarcoma co-existing with synchronous liver metastases of rectal cancer. The patient was treated by a curative hepatectomy following chemotherapy.

CASE REPORT

A 59-year-old man with a history of hypertension and

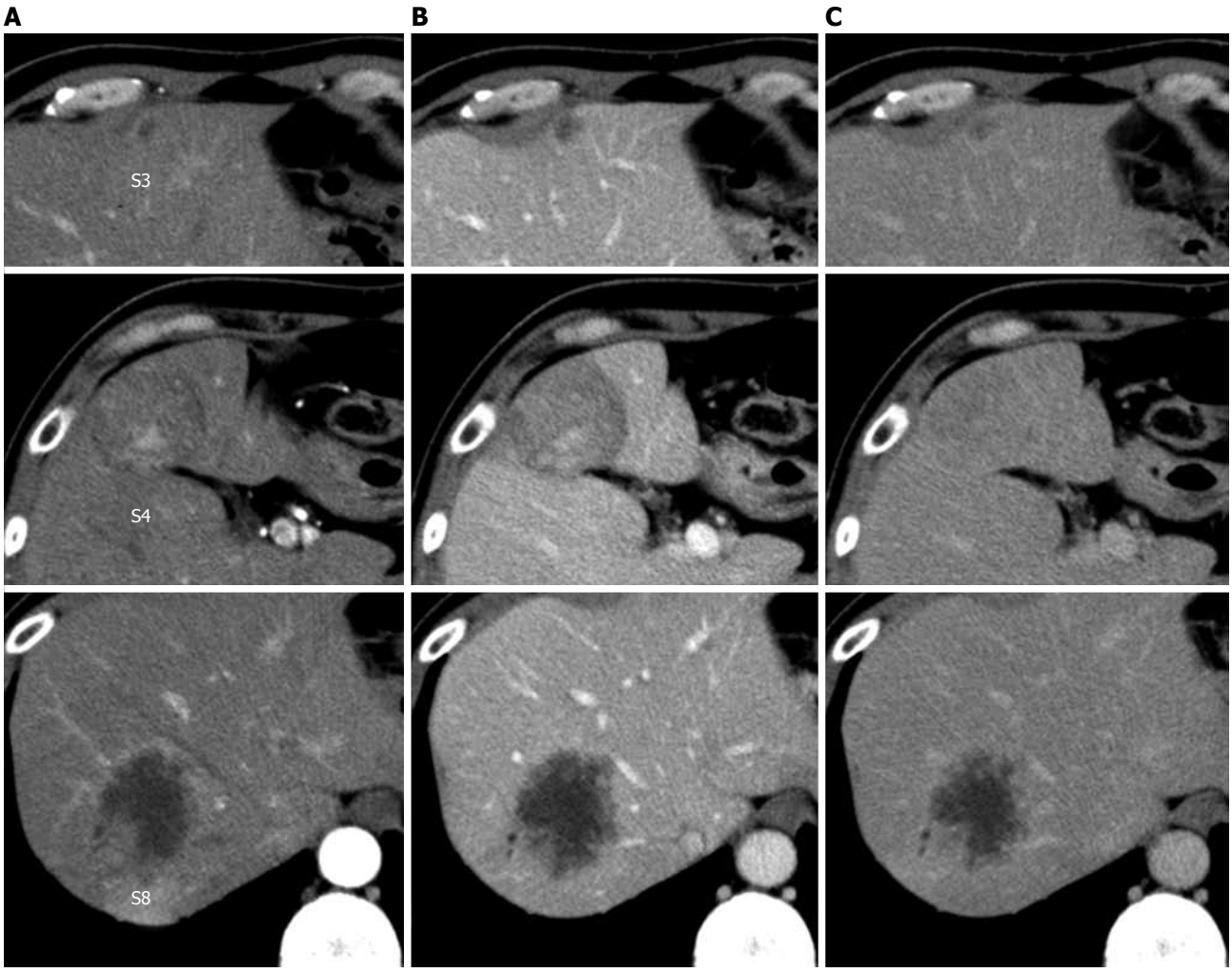


Figure 1 Dynamic computed tomography of the liver. A: Arterial phase; B: Portal phase; C: Venous phase. A hypo- or iso-dense tumor with heterogeneous enhancement in segment 4, a hypodense tumor with peripheral enhancement in segment 8, and a small hypodense tumor in segment 3.

hyperuricemia demonstrated fecal occult blood and multiple liver tumors in a comprehensive medical examination in October 2009 and was observed at our hospital. There were no remarkable physical findings. Laboratory examinations revealed a slight increase in uric acid (7.4 mg/dL). Hepatitis B and C viral markers were negative. Of the tested tumor markers, only carcinoembryonic antigen was elevated (17.9 ng/mL).

Dynamic computed tomography (CT) of the liver revealed a well-defined, hypo- or isodense tumor, 42 mm in diameter, with early enhancement and delayed washout or gradual enhancement in segment 4. Segment 8 contained an ill-defined, hypodense tumor, 52 mm in diameter, with continuous peripheral enhancement, and segment 3 contained a hypodense tumorthat was 11 mm in diameter (Figure 1).

The tumor in segment 4 appeared to be isointense throughout the dynamic phase on gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging (MRI), while the tumors in segments 3 and 8 appeared hypointense. All of the tumors appeared hypointense in pre-enhancement scans and the hepatocellular phase on T1-weighted images (Figure 2). Additionally, all of the tumors appeared hyperintense on T2-weighted images and diffusion-weighted images. Ultrasonography demonstrated that the tumors were hypo- or isoechoic heterogeneous masses without halos (Figure 3).

Colonoscopy revealed a Borrmann type 2 tumor at the rectosigmoid junction. Histology of the biopsy tissue revealed a moderately differentiated adenocarcinoma.

The patient was diagnosed with rectal cancer and multiple liver metastases. Because the simultaneous resection of the primary tumor and liver metastases would be a high-risk procedure, we performed an initial high anterior resection with D3 dissection in December 2009. A histopathological examination revealed extraserosal invasion by a moderately differentiated adenocarcinoma and metastases in 8 of 14 resected lymph nodes. A genetic analysis confirmed wild-type KRAS. The patient received chemotherapy with modified FOLFOX6 (mFOLFOX6) plus bevacizumab after the initial surgery. The liver tumor in segment 3 vanished, and the tumor in segment 8 decreased in size from 52 mm to 25 mm in diameter. However, the tumor in segment 4 increased

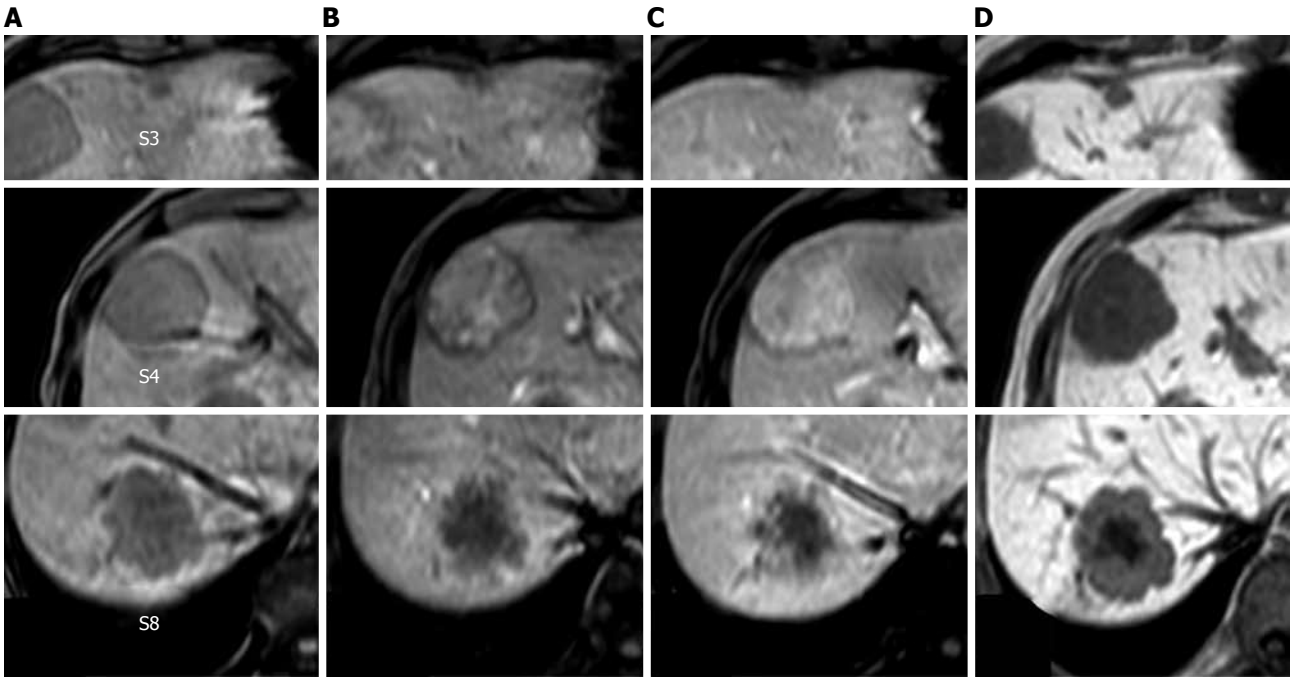


Figure 2 T1-weighted gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging. A: Pre-enhancement; B: 30 s; C: 120 s; D: Hepatocellular phase. The tumor in segment 4 showed heterogeneous enhancement in the dynamic phase. The tumors in segments 3 and 8 showed gradual peripheral enhancement.

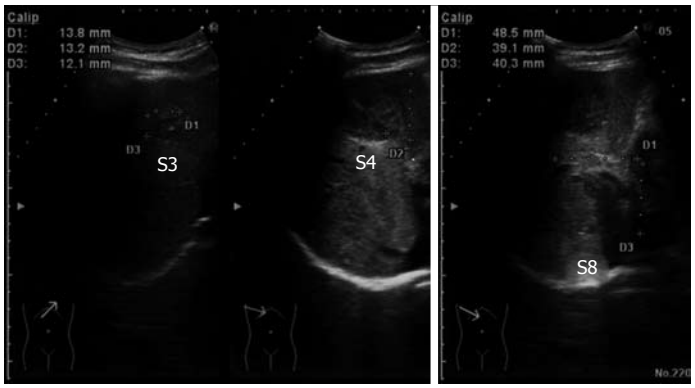


Figure 3 Ultrasonography of the abdomen. All of the tumors showed hypo- or isoechoic heterogeneous masses without halos.

in size from 42 mm to 60 mm in diameter. He then underwent seven courses of FOLFIRI plus bevacizumab, followed by a course of irinotecan plus cetuximab; the tumor in segment 4 decreased in size from 60 mm to 55 mm in diameter, and the tumor in segment 8 decreased in size from 25 mm to 22 mm in diameter (Figure 4). With the exception of the liver tumors, positron emission tomography-CT after the chemotherapy series showed no significant uptake of fluorodeoxyglucose.

The patient then underwent a central two-segment resection and a partial resection of segment 3 in September 2010. The resected specimen from the two central segments weighed 380 g, while that from segment 3 weighed 3.8 g. The tumors from segments 4, 8 and 3 measured 56 mm × 44 mm, 24 mm × 22 mm and 7 mm × 4 mm, respectively (Figure 5). A histopathological examination revealed fibrosis and calcification in the tumors from segments 3 and 8, with a few degenerated residual adenocarcinoma cells, which was compatible with rectal cancer metastasis. In contrast, the tumor from segment 4 consisted of irregular fascicles of spindle-shaped cells with eosinophilic cytoplasms and nuclear atypia (Figure 6). Chemotherapy had no apparent effect histologically. An immunohistochemical examination demonstrated that the tumor cells in segment 4 were positive for α-smooth muscle actin and desmin, and negative for CD34, S-100, c-kit and cytokeratin AE1/3 (Figure 7). Based on these findings, the tumor in segment 4 was diagnosed as a leiomyosarcoma.

The patient underwent six courses of FOLFIRI plus bevacizumab as adjuvant chemotherapy, and had no recurrence of leiomyosarcoma or rectal cancer at 16 mo after the hepatectomy.

DISCUSSION

Primary hepatic leiomyosarcoma is a particularly rare tumor. Most hepatic leiomyosarcomas are metastatic

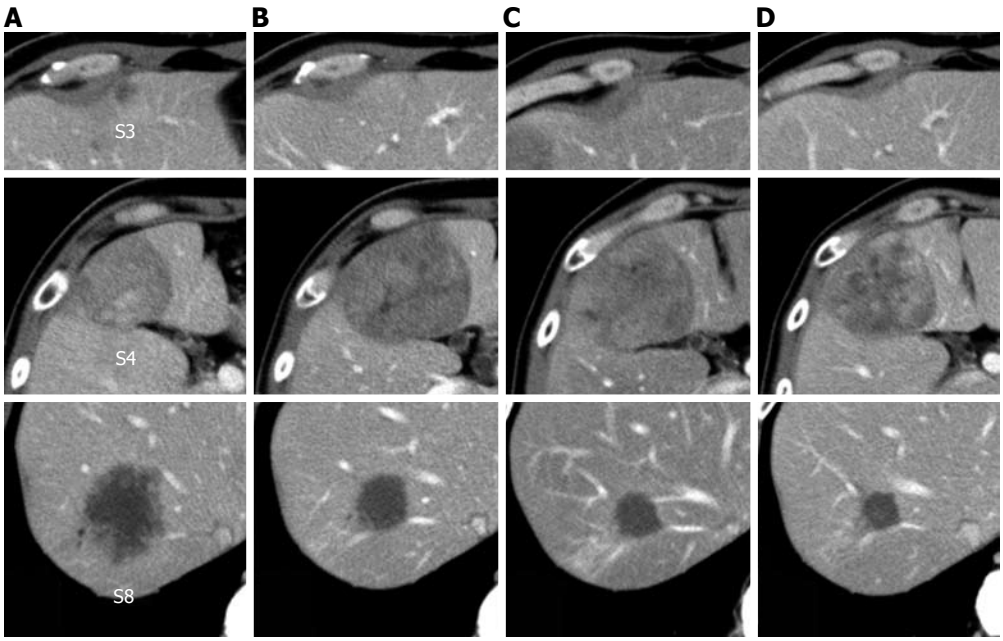


Figure 4 Change in tumor size observed using computed tomography during the clinical course. A: Before treatment; B: After six courses of modified FOLFOX6 plus bevacizumab; C: After seven courses of FOLFIRI plus bevacizumab; D: After one course of irinotecan plus cetuximab.

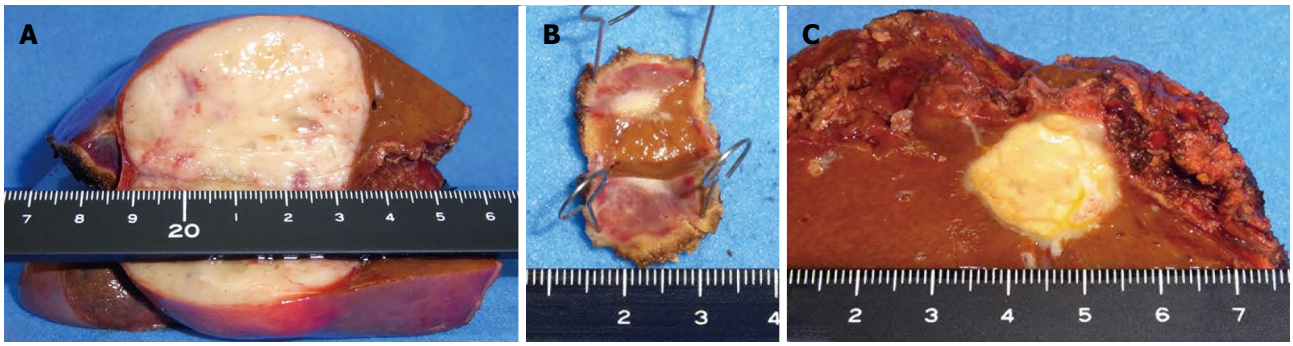


Figure 5 A macroscopic view of the cut-end surfaces of the resected specimens. A: Segment 3; B: Segment 8; C: Segment 4.

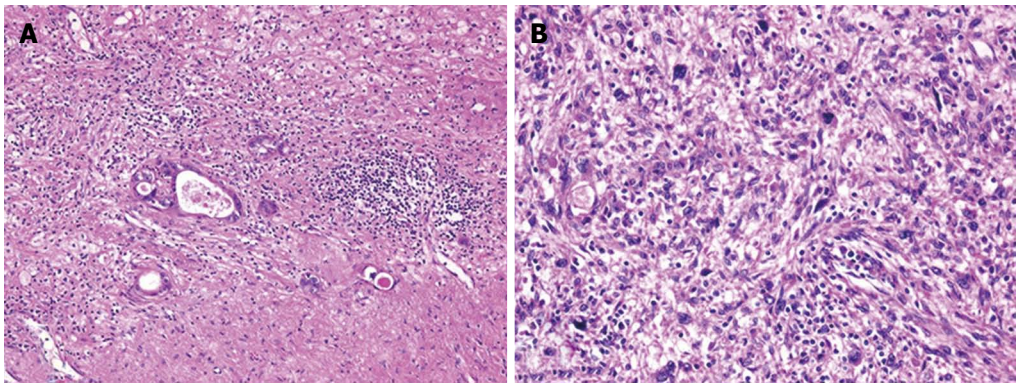


Figure 6 Histological findings of the tumors and hematoxylin and eosin staining. A: The tumor in segment 8 demonstrated fibrosis and calcification, with a few degenerated residual adenocarcinoma cells; B: The tumor in segment 4 had irregular fascicles of spindle-shaped cells with eosinophilic cytoplasm and nuclear atypia.

tumors, and the exclusion of metastatic leiomyosarcoma is therefore essential for an accurate diagnosis[3]. To our knowledge, this represents the first case report in the literature to describe a primary hepatic leiomyosarcoma co-existing with metastatic liver tumors of another origin, but the clinical and histological relevance is unclear. Surgery is considered to be the best treatment for primary hepatic leiomyosarcomas, if an R0 resection can

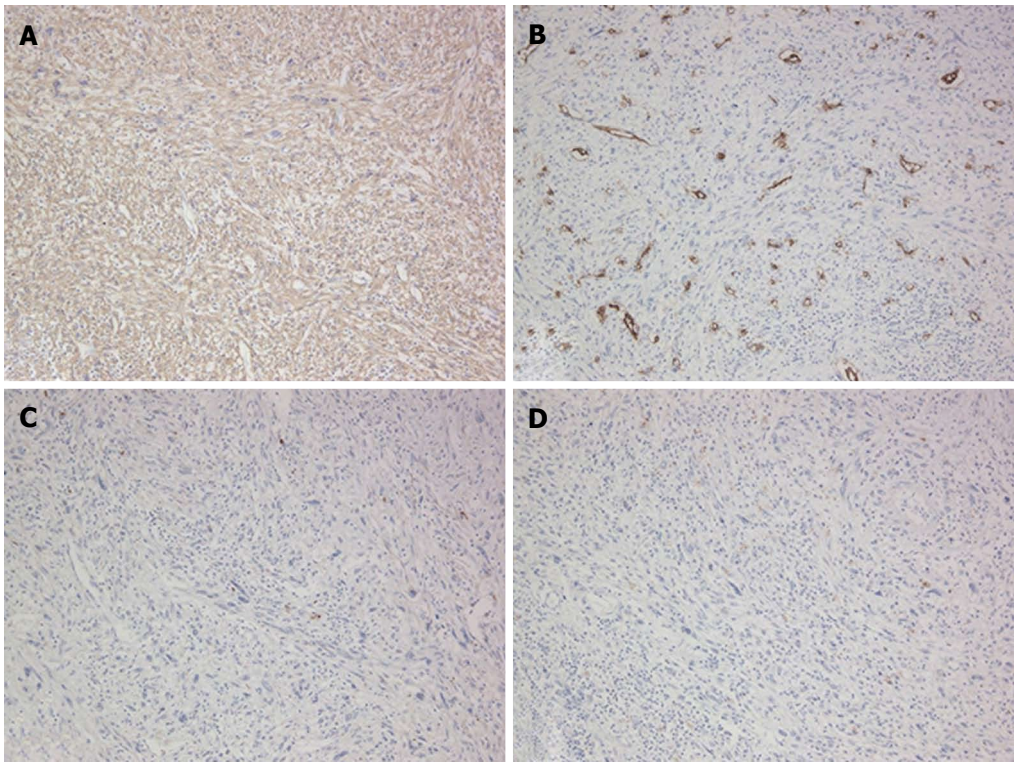


Figure 7 Immunohistochemical features of the tumor in segment 4. A: Positive for α-smooth muscle actin; B: Negative for CD34; C: Negative for S-10; D: Negative for c-kit.

be performed[3,4]. Doxorubicin has been the standard chemotherapy for soft tissue leiomyosarcomas, with gemcitabine and docetaxel or trabectedin as possible alternatives[5,6]. However, the efficacy of chemotherapy for primary hepatic leiomyosarcoma has not been confirmed. In our case, mFOLFOX6 plus bevacizumab did not inhibit the growth of the leiomyosarcoma, while FOLFIRI plus bevacizumab and irinotecan plus cetuximab did. Some studies have demonstrated the efficacy of irinotecan combined with other anticancer agents in some types of sarcomas, such as Ewing's sarcoma[7] or rhabdomyosarcoma[8], but little is known about the effect of irinotecan on leiomyosarcomas. The present case suggests that irinotecan exerted a growth-inhibiting effect on this leiomyosarcoma.

In a randomized controlled trial, Portier *et al*[9] demonstrated a disease-free survival benefit of adjuvant chemotherapy with 5-fluorouracil and leucovorin after liver resection for patients with liver metastases from colorectal cancer compared to surgery alone. In another randomized controlled trial, Nordlinger *et al*[10] demonstrated that perioperative chemotherapy with FOLFOX4 in patients with resectable liver metastases improved progression-free survival compared to surgery alone. However, adjuvant chemotherapy after hepatectomy for patients converted from unresectable liver metastases is still controversial. We selected FOLFIRI plus bevacizumab for adjuvant chemotherapy with the expectation of an effect of irinotecan on the leiomyosarcoma and because the patient developed grade 3 peripheral neuropathy with mFOLFOX6.

The preoperative diagnosis of primary hepatic leiomyosarcoma is challenging because of the non-specific nature of the symptoms and the lack of serological markers[11]. CT usually shows a well-defined hypodense heterogeneous mass with peripheral enhancement. MRI shows hypointensity on T1-weighted images and hyperintensity on T2-weighted images[12]. Some cases of hepatic leiomyosarcoma diagnosed by fine-needle aspiration cytology have been reported[13]. However, the preoperative histological diagnosis of liver tumors, especially hepatocellular carcinoma, is controversial because of the risk of needle-track seeding. In the current case, the complication of liver metastases from rectal cancer made preoperative diagnosis more difficult. In retrospect, however, the leiomyosarcoma lesion showed slightly different CT and MRI findings compared to the metastatic tumors.

In conclusion, this case suggests the potential of irinotecan to inhibit the growth of hepatic leiomyosarcomas. Irinotecan may be a chemotherapy option for hepatic leiomyosarcomas. The possibility of different histological types of tumors should be considered when planning the treatment of multiple liver tumors, especially when the tumors might have heterogeneous responses to chemotherapy.

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CASE REPORT

Duodenal obstruction following acute pancreatitis caused by a large duodenal diverticular bezoar

Ji Hun Kim, Jae Hyuck Chang, Sung Min Nam, Mi Jeong Lee, Il Ho Maeng, Jin Young Park, Yun Sun Im, Tae Ho Kim, Il Young Park, Sok Won Han

Ji Hun Kim, Jae Hyuck Chang, Sung Min Nam, Mi Jeong Lee, Il Ho Maeng, Jin Young Park, Yun Sun Im, Tae Ho Kim, Sok Won Han, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul 137-701, South Korea
Il Young Park, Department of Surgery, College of Medicine, The Catholic University of Korea, Seoul 137-701, South Korea
Author contributions: Kim JH and Chang JH made substantial contributions in reviewing the case and compiling the data; Kim JH drafted the manuscript; Park IY performed the operation; Nam SM, Lee MJ, Maeng IH, Park JY, Im YS, Kim TH, and Han SW contributed to the discussion and reviewed the manuscript.
Correspondence to: Jae Hyuck Chang, MD, PhD, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, 327 Sosa-ro, Wonmi-gu, Bucheon-si, Gyeonggi-do 420-717, Seoul 137-701, South Korea. wwjjaang@catholic.ac.kr
Telephone: +82-32-3402227 Fax: +82-32-3402255
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Abstract

Bezoars are concretions of indigestible materials in the gastrointestinal tract. It generally develops in patients with previous gastric surgery or patients with delayed gastric emptying. Cases of periampullary duodenal diverticular bezoar are rare. Clinical manifestations by a bezoar vary from no symptom to acute abdominal syndrome depending on the location of the bezoar. Biliary obstruction or acute pancreatitis caused by a bezoar has been rarely reported. Small bowel obstruction by a bezoar is also rare, but it is a complication that requires surgery. This is a case of acute pancreatitis and subsequent duodenal obstruction caused by a large duodenal bezoar migrating from a periampullary diverticulum to the duodenal lumen, which mimicked pancreatic abscess or microperforation on abdominal computerized tomography. The patient underwent surgical removal of the bezoar and recovered completely.

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Key words: Bezoar; Diverticulum; Pancreatitis; Duodenal obstruction

Peer reviewers: Juhani Sand, MD, PhD, Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, Teiskontie 35, 33521 Tampere, Finland; Ross Cyril Smith, Professor, Department of Surgery, University of Sydney, Suite 5 Level 5 North Shore Private Hospital, StLeonards 2065, Australia

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INTRODUCTION

Bezoars are concretions of foreign bodies found in the gastrointestinal tract. They are usually detected after gastric operations because of reduced gastric motor activity and delayed gastric emptying[1]. Bezoars in a periampullary duodenal diverticular without previous surgery are rare. The presence of bezoars may or may not be accompanied by gastrointestinal manifestations, such as epigastric pain and postprandial fullness. Major complications of bezoars include gastritis, gastric ulcer, gastric perforation, and intestinal obstruction[2]. Small bowel obstruction is a rare complication usually due to the migration of gastric bezoars. It could also be caused by formation of primary bezoars in the small bowel in association with underlying diseases such as diverticulum, stricture or tumor[3]. Acute pancreatitis secondary to a bezoar is rare, but can present as a possible complication[4]. However, duodenal obstruction by a diverticular bezoar following acute pancreatitis

has not been reported.

We report a case of acute pancreatitis and subsequent duodenal obstruction caused by a large duodenal bezoar migrating from periampullary diverticulum to duodenal lumen, which was difficult to diagnose by radiological study.

CASE REPORT

A 75-year-old female visited our hospital with epigastric pain, nausea and vomiting for 4 d. She was diagnosed with diabetes 15 years ago and was taking oral hypoglycemic agents for the control of blood sugar level. There were no specific findings from her social history. Her blood pressure on admission was 130/80 mmHg, pulse rate 70/min, respiratory rate 20/min, and body temperature 36.4 ℃. On physical examination, she appeared acutely ill, with a soft abdomen but with tenderness in the epigastric area and right upper quadrant. Complete blood count test showed a leukocyte count of 10 340/mm^3, a hemoglobin level of 13.1 g/dL, and a platelet count of 174 000/mm^3. Blood chemistry showed total protein 7.1 g/dL, albumin 4.3 g/dL, total bilirubin 1.1 mg/dL, aspartate aminotransferase 491 IU/L, alanine aminotransferase 375 IU/L, alkaline phosphatase 154 IU/L, γ-glutamyl transpeptidase 213 IU/L, amylase 1730 IU/L, and lipase over 300 IU/L. Abdominal computerized tomography (CT) taken on the day of admission revealed a 5 cm sized subtle rim enhancing mass with air bubbles in the pancreatic head which raised the suspicion of an air forming abscess of the pancreatic head or a duodenal diverticulum of the second duodenal portion (Figure 1A and B). Following this, magnetic resonance cholangiopancreatography (MRCP) showed no evidence of abnormality in the common bile duct or pancreatic duct. The medical treatments including intravenous fluids, parenteral alimentation, and antibiotics were started, and abdominal pain improved thereafter. Additionally, the elevated serum transaminase and pancreatic enzymes progressively improved. However, nine days after admission, the patient's body temperature rose to 40.4 ℃, her blood pressure was 80/50 mmHg, leukocyte count 47 620/mm^3, erythrocyte sedimentation rate 66 mm/h, and C-reactive protein 166 mg/dL. The follow-up abdominal CT revealed aggravation of duodenal wall thickening and stranding of the surrounding soft tissues with some tiny extraluminal air bubbles indicating possible micro-perforation (Figure 1C). She received fluid resuscitation through rapid intravenous administration of crystalloid fluid and vasopressor support, and antibiotic therapy with meropenem was started for the management of septic shock. After vigorous treatment, the patient's state became stabilized, but more than 1500 cc of dark-colored fluid was drained through the Levin tube per day. Esophagogastroduo- denoscopy (EGD) was performed for suspected duodenal obstruction. On entering the duodenum, a huge dark yellowish mass was found obstructing the lumen of the second portion of the duodenum. This mass was thought

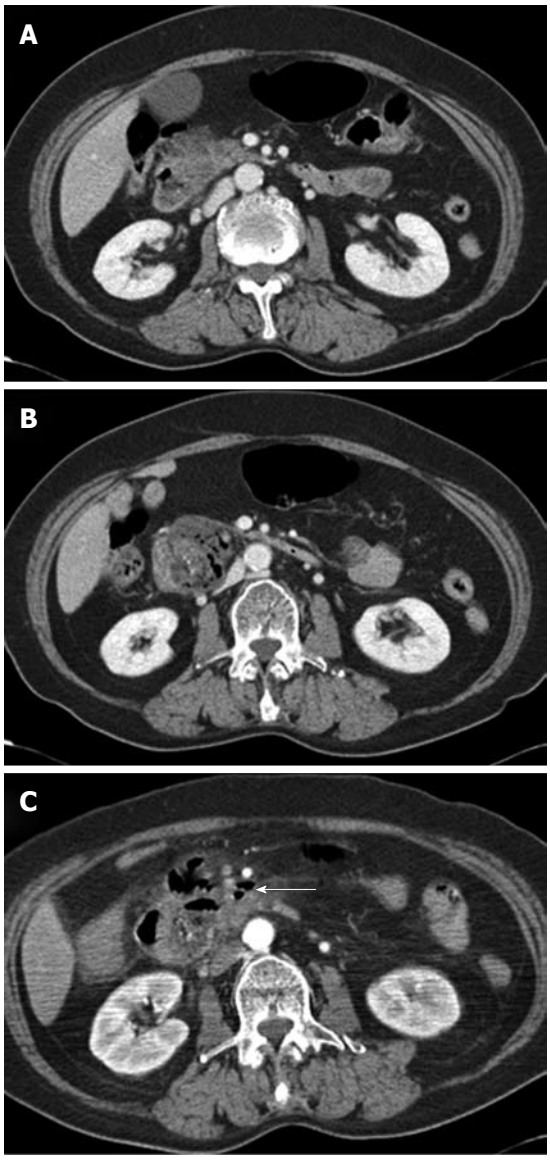


Figure 1 Abdominal computed tomography findings. A: The pancreas head showed swelling with irregular contour of the pancreatic margin and mild peripancreatic infiltration; B: There is a 5 cm size subtle rim enhancing mass with air bubbles, indicating acute diverticulitis in the second duodenal portion or air forming abscess of the pancreatic head; C: After nine days, a few extraluminal air bubbles (arrow) suspicious for microperforation were found.

to be originated from the large periampullary diverticulum and migrated toward the distal portion (Figure 2A). A large broad based inflamed diverticulum was observed, with small secondary diverticula corresponding to the extraluminal air bubbles seen on the follow-up abdominal CT. Endoscope was not able to pass through because of luminal obstruction. We tried to extract the bezoar with the help of a basket and a net, but it was unsuccessful as the bezoar was too hard and big (Figure 2B). On eighteenth hospital day, when the clinical symptoms and the laboratory findings of acute pancreatitis and septic shock had been improved, the patient was transferred to the department of general surgery for operation. A gastrotomy was performed and a huge bezoar (5 cm × 2.5 cm) was removed through the antrum by manually push-

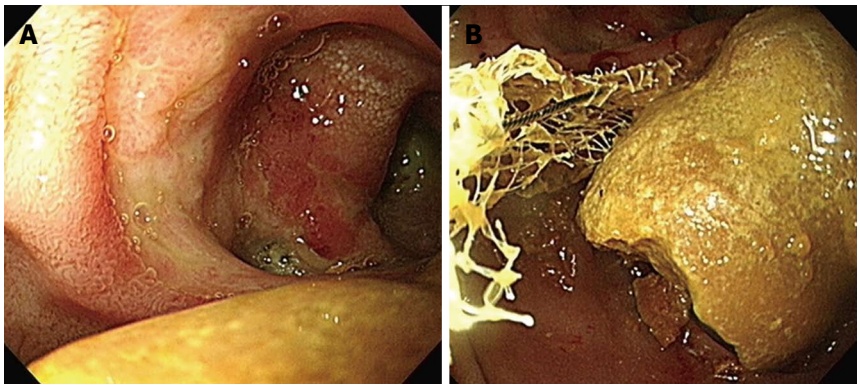


Figure 2 Endoscopic findings. A: There is a huge luminal obstructing bezoar near the duodenal diverticulum; B: The bezoar was too large to be captured and removed by an endoscopic basket or net.

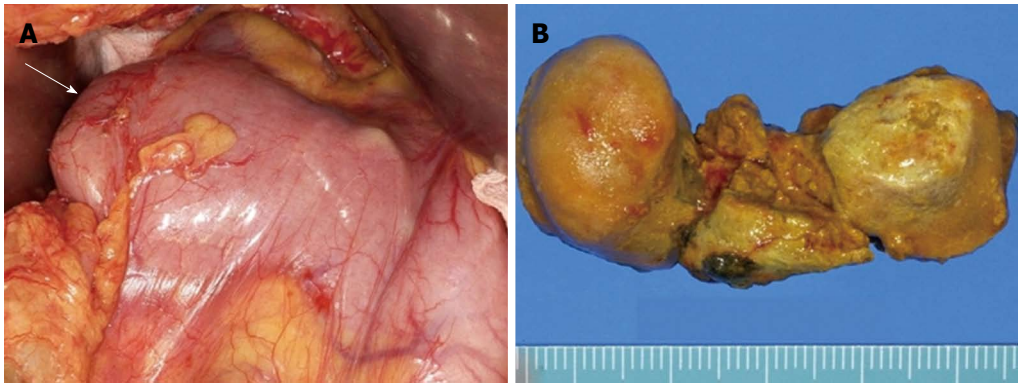


Figure 3 Surgical findings. A: Obstructed duodenum with impacted huge bezoar (arrow); B: Surgical specimen of the divided bezoar, 5 cm long, 2.5 cm in diameter.

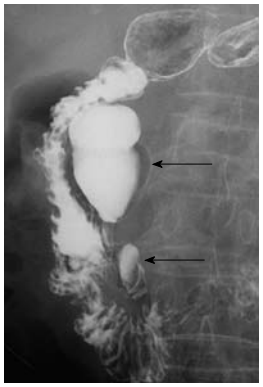


Figure 4 Upper gastrointestinal series show two periampullary diverticula (arrows) in the second duodenal portion.

ing the duodenal bezoar up into the stomach (Figure 3). The patient was discharged on the 6th postoperative day. During the 2-mo follow-up, the patient did not complain of any discomfort and two periampullary diverticula were seen in the upper gastrointestinal series (Figure 4).

DISCUSSION

Bezoars are composed of vegetable matter (phytobezoar), hair (trichobezoar), or more unusual materials[1]. They are usually related to previous gastric operations, such as vagotomy or partial gastrectomy, which reduce gastric motor activity and delay gastric emptying. In one report, 70% of patients with bezoar had undergone previous surgery[2]. Bezoars also can be induced by gastroparesis caused by hypothyroidism or diabetes mellitus, and poor mastication or ingestion of indigestible materials[3].

Most bezoars occur in the stomach, but may be encountered elsewhere, including the small bowel and even the esophagus and rectum. Clinical manifestations vary depending on the location of the bezoar from showing no symptom to acute abdominal syndrome, such as, epigastric distention, abdominal pain, and acid regurgitation. Major complications of bezoars include intestinal obstruction, gastric perforation, gastric ulcer, and gastritis[5]. Small bowel obstruction is a severe complication requiring surgery[6]. Small bowel obstructions were usually due to migration of gastric bezoars, but it could also be caused by primary bezoars formed in the small bowel in association with stricture, tumor or diverticulum[3]. Biliary obstruction or acute pancreatitis caused by bezoars has seldom been reported[4,7,8]. In our case, a large duodenal bezoar had originated from an inflamed periampullary diverticulum, causing acute pancreatitis. This was then followed by a spontaneous migration of the bezoar, resulting in a duodenal obstruction and septic shock. The duodenal obstruction due to large gallstone migrated through a cholecysto-enteric fistula can give a similar

radiological and endoscopic appearance as our case[9]. However, there was no cholecysto-duodenal fistula or air in the gallbladder in the present case.

Although duodenal bezoars are rare, they can develop in patients with previous gastric surgery[8], deformed duodenal bulb[10], superior mesenteric artery syndrome[11], and duodenal diverticulum[12] as in our case. Most cases of pancreatitis caused by duodenal bezoars are trichobezoars also known as the "Rapunzel syndrome"[4]. This was a case of having unusually long hairballs that extend from the stomach into the jejunum or ileum. It may cause extrinsic compression of the ampulla, common bile duct or pancreatic head. It is thought that bezoars in the periampullary diverticulum may also cause acute pancreatitis through similar mechanisms[13].

Several studies suggested that patients with periampullary diverticulum had higher frequencies of pancreatitis. It is still controversial whether or not pancreatitis is induced by periampullary diverticulum per se. Periampullary diverticulum is associated with choledocholithiasis, and it predispose to gallstone pancreatitis[14]. Our patient showed no evidence of stones in the common bile duct on abdominal CT and MRCP, thus acute pancreatitis did not result from bile duct stones. In fact, the large bezoar had caused compression of major ampulla leading to pancreatitis.

Contrast-enhanced abdominal CT is the most useful tool for diagnosis of duodenal diverticulitis, but it can be misinterpreted as acute pancreatitis or its complications (phlegmon, pseudocyst, abscess), cystic pancreatic head neoplasms, and peripancreatic lymphadenopathy[15]. In our case, the enhanced CT showed findings that were suspicious for pancreatic abscess or microperforation of duodenal diverticulum adjacent to the pancreatic head. After EGD, the condition was recognized as periampullary diverticulitis with secondary diverticula inside. Endoscopy is an important diagnostic modality for diagnosis of diverticular diseases. The side-viewing endoscopy can be beneficial compared to the forward-viewing endoscopy, because acute diverticulitis causes mucosal swelling and narrowing of the diverticular orifice that further hampers the diagnostic yield of a forward-viewing endoscopy[16].

Bezoars are treated by endoscopy or surgery. If the bezoar is large, fragmentation is tried with the use of overtube, baskets, lithotripsic equipment, paraffin, cellulose, acetylcysteine, Coca-Cola lavage, and even lasers. If the bezoar is accompanied by complications such as small bowel obstruction, gastric perforation or gastric hemorrhage, the patient needs urgent treatment, such as surgery[3].

In conclusion, we report a case of a large duodenal bezoar, which had originated from the periampullary diverticulum, causing acute pancreatitis, with subsequent duodenal obstruction by migration. Complications caused by duodenal bezoar, in particular, pancreatitis and subsequent duodenal obstructions are rare, but a high index of suspicion is required for the correct diagnosis and proper management.

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Atsushi Nakajima, Professor, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

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George Papatheodoridis, MD, Associate Professor in Medicine and Gastroenterology, 2nd Department of Internal Medicine, Athens University Medical School, Hippokration General Hospital of Athens, 114 Vas. Sophias Ave., 115 27 Athens, Greece

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Yoshihisa Takahashi, MD, Department of Pathology, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan

Dr. Tamara Vorobjova, MD, PhD, Scimed Senior Researcher in Immunology, Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila, 19, 51014 Tartu, Estonia

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Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

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Phoenix, AZ 85001, United States

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Lake Tahoe, NV 89101, United States

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PancreasFest 2012
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September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
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Dnepropetrovsk, Ukraine

November 4-8, 2012
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San Francisco, CA 94101, United States

November 9-13, 2012
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Boston, MA 02298, United States

December 1-4, 2012
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Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
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Acknowledgments

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Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

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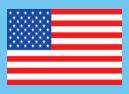
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EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
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Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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EDITORIAL

Peritoneal carcinomatosis of colorectal origin: Incidence, prognosis and treatment options

Yvonne LB Klaver, Valery EPP Lemmens, Simon W Nienhuijs, Misha DP Luyer, Ignace HJT de Hingh

Yvonne LB Klaver, Simon W Nienhuijs, Misha DP Luyer, Ignace HJT de Hingh, Department of Surgical Oncology, Catharina Hospital Eindhoven, Michelangelolaan 2, 5623 EJ Eindhoven, The Netherlands
Valery EPP Lemmens, Department of Public Health, Erasmus Medical Centre Rotterdam, Dr Molewaterplein 50, 3015 CE Rotterdam, The Netherlands
Author contributions: Klaver YLB, Lemmens VEPP, Nienhuijs SW, Luyer MDP and de Hingh IHJT designed the manuscript; Klaver YLB, Lemmens VEPP and de Hingh IHJT drafted the manuscript and made the final approval; Klaver YLB and Nienhuijs SW acquired the data; Lemmens VEPP analyzed the data; Nienhuijs SW, Luyer MDP and de Hingh IHJT revised the manuscript.
Correspondence to: Dr. Ignace HJT de Hingh, MD, PhD, Department of Surgical Oncology, Catharina Hospital Eindhoven, Michelangelolaan 2, 5623 EJ Eindhoven, The Netherlands. ignace.d.hingh@cze.nl
Telephone: +31-40-2399111 Fax: +31-40-2455035
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Abstract

Peritoneal carcinomatosis (PC) is one manifestation of metastatic colorectal cancer (CRC). Tumor growth on intestinal surfaces and associated fluid accumulation eventually result in bowel obstruction and incapacitating levels of ascites, which profoundly affect the quality of life for affected patients. PC appears resistant to traditional 5-fluorouracil-based chemotherapy, and surgery was formerly reserved for palliative purposes only. In the absence of effective treatment, the historical prognosis for these patients was extremely poor, with an invariably fatal outcome. These poor outcomes likely explain why PC secondary to CRC has received little attention from oncologic researchers. Thus, data are lacking regarding incidence, clinical disease course, and accurate treatment evaluation for patients with PC. Recently, population-based studies have revealed that PC occurs relatively frequently among patients with CRC. Risk factors for developing PC have been identified: right-sided tumor, advanced T-stage, advanced N-stage, poor differentiation grade, and younger age at diagnosis. During the past decade, both chemotherapeutical and surgical treatments have achieved promising results in these patients. A chance for long-term survival or even cure may now be offered to selected patients by combining radical surgical resection with intraperitoneal instillation of heated chemotherapy. This combined procedure has become known as hyperthermic intraperitoneal chemotherapy. This editorial outlines recent advancements in the medical and surgical treatment of PC and reviews the most recent information on incidence and prognosis of this disease. Given recent progress, treatment should now be considered in every patient presenting with PC.

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Key words: Colorectal cancer; Peritoneal carcinomatosis; Hyperthermic intraperitoneal chemotherapy; Chemotherapy; Prognosis

Peer reviewers: Dr. Marek Bebenek, MD, PhD, Department of Surgical Oncology, Regional Comprehensive Cancer Center, pl. Hirszfelda 12, 53-413 Wroclaw, Poland; Jai Dev Wig, MS, FRCS, Former Professor and Head, Department of General Surgery, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

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INTRODUCTION

Peritoneal carcinomatosis (PC) secondary to colorectal

cancer (CRC) is characterized by the development of solid tumor deposits on the peritoneal surface[1]. Cell shedding from the primary tumor is thought to be responsible for these peritoneal deposits, which may occur spontaneously or as a result of spillage during surgical procedures. Attachment of tumor cells to peritoneal mesothelial cells involves neoangiogenesis and is mediated by several growth factors[2]. Tumor implantation and growth may lead to invasion of any organ or structure that is covered by peritoneum.

Common sites for peritoneal implants are the omentum, mesentery, bowel surface, pouch of Douglas, right paracolic gutter, and diaphragm[3,4]. Patients initially present with nonspecific symptoms such as abdominal discomfort, nausea, weight loss, cachexia, and fatigue; however, these symptoms are often indistinguishable from more general features of malignant disease. The tumor growth on intestinal surfaces and associated fluid accumulation eventually result in signs of bowel obstruction[5] and incapacitating volumes of ascites[6,7]. Previously, providers were reluctant to treat these patients because of their extremely poor prognosis and invariably fatal outcomes[8-10]. Therefore, PC resulting from CRC had received little attention from oncologic researchers. Hence, data was lacking regarding incidence, clinical disease course, and accurate treatment evaluation for patients with PC.

Since the 1980s, PC research has attracted renewed interest because of the observation that a subgroup of patients presents solely with peritoneal tumor implants without systemic metastases[11,12]. This finding spurred the development of aggressive surgical treatment modalities which combined radical cytoreductive surgery with intraperitoneal chemotherapy[13-17]. With this approach, prolonged overall survival and even disease cure have been reported[8,18-26]. Here, we will discuss the recent advances in the characterization and treatment of these patients.

INCIDENCE OF PC

The extent of peritoneal disease is frequently underestimated by imaging modalities[27], and the presence of peritoneal involvement often remains unknown until a laparotomy is performed. This uncertainty in preoperative diagnosis results from the low sensitivity and specificity of imaging techniques such as abdominopelvic ultrasound and computed tomography. The small size of tumor deposits (typically less than 1 cm) negatively affects sensitivity[27-30]. Furthermore, peritoneal spread of tumor cells characteristically follows the anatomic outline of normal abdominal structures, making radiologic detection more challenging.

Underestimation of PC due to poor preoperative imaging diagnostics, combined with the aforementioned lack of interest, likely explains the virtual absence of data on PC incidence. Most available data were retrieved from single hospital-based studies that reported incidence of PC encountered during laparotomy. In the largest study, which included 2756 patients with CRC, 214 (8%) patients were diagnosed with synchronous PC and 135 (5%) with metachronous disease[10]. Two older studies, also single hospital-based, reported that 10% to 15% of patients with colon cancer presented with PC[9,31]. Recently, two population-based studies reported the incidence of synchronous PC in The Netherlands (4.8%) and in Sweden (4.3%)[32,33]. Risk factors for developing PC include right-sided tumor, advanced T-stage, advanced N-stage, poor differentiation grade, and younger age at diagnosis.

In clinical studies, metachronous PC is reported in 4% to 12% of patients following curative resection for colon cancer and in 2% to 19% of patients following curative resection for rectal cancer[34]. In patients undergoing repeat procedures for CRC following primary curative resection, 21% to 44% of patients are diagnosed with peritoneal tumor deposits[35,36]. In autopsy studies, PC is found in up to 40% of patients who die from colorectal carcinoma[37,38]. On a population level, 4.2% of Swedish patients with CRC developed metachronous PC following initial treatment[33]. Risk factors for developing metachronous PC are similar to those for synchronous PC, but also include initial emergency procedures and non-radical initial tumor resection[33].

TREATMENT

Systemic treatment

Few studies have been published describing the effectiveness of systemic chemotherapy in patients with PC. Due to an inability to accurately measure tumor load and treatment response, patients with peritoneal tumors usually do not meet the inclusion criteria for randomized trials[39]. The few studies describing chemotherapeutic treatment response focus on systemic 5-fluorouracil (5-FU) and leucovorin using retrospective analysis. The results invariably show a disappointing response to systemic treatment and a poor prognosis compared to other metastatic sites. A French prospective multicenter study of 118 patients with PC of colorectal origin showed a median survival of only 5.2 mo[40]. In a large series of CRC patients, which included 392 patients with peritoneal involvement, Jayne *et al*[10] showed a median survival of 7 mo. Chu *et al*[9] reported a median survival of 6 mo in a series of 45 patients who were treated primarily with 5-FU and leucovorin. A sub-analysis by Köhne *et al*[41] of patients with PC treated with 5-FU-based therapy showed a median survival of 7.7 mo. Slightly better results were reported by Bloemendaal *et al*[8], who described 50 patients with PC but without hematogenous metastases who were treated with systemic chemotherapy and palliative surgery. Their overall median survival was 12.6 mo, with a 2-year survival rate of approximately 18%[8,24]. However, selection bias probably explains these findings, because these patients were initially referred for hyperthermic intraperitoneal chemoperfusion (HIPEC)-

treatment but eventually randomized to the study control group. It is conceivable that these patients were healthier and their PC disease was more limited compared to the average PC patient.

A few studies have aimed to describe the effect of newer chemotherapeutic combinations, such as oxaliplatin plus irinotecan[8,42,43]. Results are conflicting and require careful interpretation. Many of these studies were performed to compare systemic treatment with surgical treatment. Selection bias may play a role in these studies because only patients in good condition with limited disease and without systemic metastases were eligible. Nevertheless, the median survival of 23 mo, as described by Elias *et al*[18] and obtained with modern systemic chemotherapy, is remarkable and dispels the notion that PC is chemotherapy-resistant. A similar conclusion may be drawn from the only population-based study to investigate this topic thus far. From 1995 to 2008, the administration of chemotherapy to patients with PC gradually increased, from 16% to 46% ($P = 0.001$), with the treatment rate rising to 64% for younger patients[41]. However, a survival benefit was only apparent after 2005 when modern chemotherapy schedules were introduced[44].

Introduction of targeted therapies, including monoclonal antibodies specifically targeted to epidermal growth factor receptor and vascular endothelial growth factor, has resulted in a significantly increased survival among patients with metastasized CRC[41,45,46]. Although these agents are now routinely included in the treatment of patients with stage Ⅳ disease, only one small retrospective study has evaluated the effect of adding targeted therapies in patients with PC; a survival of 22.4 mo was observed when biologicals were added to first line of treatment in this patient group[47].

Cytoreductive surgery and intraperitoneal chemotherapy

The observation that some patients present with PC in the absence of systemic metastases has led to the hypothesis that PC results from locoregional spread rather than systemic metastasis. This belief has encouraged surgical oncologists to examine possibilities for locoregional therapies. In the 1980s and 1990s, physicians and researchers developed new treatment strategies consisting of aggressive cytoreductive surgery plus intraperitoneal chemotherapy, often combined with hyperthermia.

Surgical procedures invariably start with a careful and systematic abdominal exploration and registration of the extent of peritoneal disease. The abdomen is divided in 13 regions and for each region, the number and size of tumor deposits are assessed and recorded. The sum of these scores represents the peritoneal cancer index (PCI), which ranges from 0 to 39. For PC resulting from CRC, a PCI score of 15 or more is generally accepted as exclusion criterion for HIPEC. In The Netherlands, the simplified PCI (sPCI) is commonly used to describe the PC involvement of 9 regions of the abdomen[24]. The PCI and sPCI are well-known predictive outcome indices for patients undergoing cytoreductive surgery and perioperative chemotherapy[48,49].

During cytoreductive surgery, surgeons attempt to remove all visible tumor deposits from the peritoneal surface. To achieve a radical resection, resection of grossly involved organs may be required. Additionally, peritonectomy may be performed[15]. Resection completeness is recorded using the completeness of cytoreduction score (CCR). A CCR-0 score indicates that no macroscopic peritoneal tumor remains following cytoreduction. A CCR-1 score occurs when tumor nodules less than 2.5 mm persist following cytoreduction. Residual disease measuring 2.5 mm to 2.5 cm is scored as CCR-2. A CCR-3 score indicates the presence of tumor nodules greater than 2.5 cm, or a confluence of unresectable tumor nodules at any site within the abdomen or pelvis[50]. Alternatively, the R1-R2a-R2b scoring system classifies R1 as no macroscopic residual tumor, R2a as macroscopic residual disease less than 2.5 mm, and R2b as tumor deposits greater than 2.5 mm[24]. Treatment outcomes are poorer in the presence of residual tumor following optimal cytoreduction, particularly for residual tumor diameters exceeding 2.5 mm. It is hypothesized that 2.5 mm is the maximum penetration depth of chemotherapeutic agents[48].

After macroscopically complete cytoreduction, intraperitoneal chemotherapy is administered to eradicate microscopic disease. This chemotherapy can be administrated immediately following surgery in the operating room, usually in combination with HIPEC or on postoperative days 1 to 5 (early postoperative intraperitoneal chemotherapy). HIPEC perfusion may be performed with a closed abdomen, or with an open "coliseum technique". Chemotherapeutic agents and doses vary widely between centers worldwide, with mitomycin C and oxaliplatin being the most frequently used agents.

Only one completed phase Ⅲ randomized trial investigating the outcome of surgical intraperitoneal treatment has been published to date. Verwaal *et al*[24,25,51] reported a significant increase in median overall survival among patients treated with cytoreductive surgery and HIPEC, as compared to patients receiving standard palliative care using systemic 5-FU and leucovorin. The promising outcomes observed in this study have convinced many surgeons to accept this technique as standard of care for selected patients with PC, and HIPEC treatment is now offered in specialized centers all over the world. However, this study was heavily criticized for not including a control group of patients receiving cytoreductive surgery only. Therefore, it remains unclear whether cytoreductive surgery and HIPEC are both required to improve survival, or if the observed benefit was due to a single component[52]. Ideally, these questions should be addressed in randomized trials. However, this type of study has proven difficult to implement, as demonstrated by a phase Ⅲ trial in France that failed to enroll enough patients due to patient dissatisfaction

with randomization[53]. Recently, investigations of cytoreductive surgery and HIPEC in PC animal models have provided a sound scientific rationale for the application of intraperitoneal chemotherapy in conjunction with cytoreductive surgery[54-56], although the additional value of hyperthermia is questionable[54].

The best available clinical evidence now comes from multi-institutional registries[18-23]. These data require careful interpretation, as surgeon experience, technique and perioperative care differs widely between institutions[57]. However, reported median survival rates of up to 63 mo following cytoreductive surgery and HIPEC with limited postoperative morbidity and mortality[58,59] suggest that treatment should be considered in all patients with PC secondary to CRC.

In conclusion, PC of colorectal origin was formerly considered untreatable, with an extremely poor prognosis. Recent treatment advances using modern systemic chemotherapy or cytoreductive surgery combined with HIPEC have improved patient outcomes. In our opinion, treatment should be considered for every patient presenting with PC due to CRC. However, further understanding of PC pathogenesis, optimal diagnostics and treatment requires ongoing experimental and clinical research.

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FIELD OF VISION

Diagnosis of Zollinger-Ellison syndrome: Increasingly difficult

Tetsuhide Ito, Guillaume Cadiot, Robert T Jensen

Tetsuhide Ito, Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan
Guillaume Cadiot, Hepato-Gastroenterology Service, Centre hospitalier universitaire de Reims, Hospital Robert Debre, F-51092 Reims, France
Robert T Jensen, Digestive Diseases Branch, National Insitutes of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, United States
Author contributions: Ito T, Cadiot G and Jensen RT collected the materials and wrote the manuscript.
Correspondence to: Dr. Robert T Jensen, Digestive Diseases Branch, National Insitutes of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Building 10, Room 9C-103, Bethesda, MD 20892,
United States. robertj@bdg10.niddk.nih.gov
Telephone: +1-301-4964201 Fax: +1-301-4020600
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Abstract

In the present paper the increasing difficulty of diagnosis of Zollinger-Ellison syndrome (ZES) due to issues raised in two recent papers is discussed. These issues involve the difficulty and need to withdraw patients suspected of ZES from treatment with Proton Pump Inhibitors (omeprazole, esomeprazole, lansoprazole, rabeprazole, pantoprazole) and the unreliability of many gastrin radioimmunoassays. The clinical context of each of these important issues is reviewed and the conclusions in these articles commented from the perspective of clinical management.

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Key words: Zollinger-Ellison syndrome; Gastrinoma; Hypergastrinemia; Secretin test; Serum gastrin; Gastrin; Neuroendocrine tumor

Peer reviewers: Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, 5901 E. Seventh Str., Long Beach, CA 90822, United States; Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of General Medicine 2 Unit, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University Hospital of Pisa, University of Pisa, Via Roma 67, 56124 Pisa, Italy

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INVITED COMMENTARY ON HOT ARTICLES

Two recent papers by Poitras *et al*[1] and Rehfeld *et al*[2] call attention to two areas that are making it more difficult to diagnosis Zollinger-Ellison syndrome (ZES). In this short review after listing the papers and their abstracts, the importance of these two issues will be briefly commented on.

Background: General

ZES is a clinical syndrome due to the ectopic secretion of gastrin by a neuroendocrine tumor (gastrinoma), located primarily in the duodenum (60%-80%) or pancreas (10%-40%), resulting in gastric acid hypersecretion, which if left untreated results in refractory peptic ulcer disease, severe gastroesophageal reflux disease, diarrhea and finally death, primarily due to the complications of the refractory peptic ulcer disease[3-6]. The diagnosis of ZES, like the diagnosis of other ectopic hormonal pancreatic endocrine syndromes, historically requires the demonstration of inappropriate release of the hor-

mone and evidence of hormonal hypersecretion[3,6-8]. In the case of ZES this requires the demonstration of inappropriate gastrin release by demonstrating fasting hypergastrinemia in the presence of gastric acid hypersecretion[3,6-10]. This is clinically most frequently accomplished by demonstrating the inappropriate presence of fasting hypergastrinemia when gastric fluid is acidic with a pH $\leqslant$ 2 is present or gastric hypersecretion is present [> 15 mEq/h basal acid output (no previous gastric acid reducing surgery), > 5 mEq/h (if gastric acid reducing surgery)][7,8,11-14]. The combination of fasting hypergastrinemia and elevated gastric acid secretion are required for ZES diagnosis because numerous unrelated conditions can cause one or the other of these alone[5,6,8,10,14]. The most frequent cause of fasting hypergastrinemia is physiological hypergastrinemia (also called appropriate hypergastrinemia) due to the presence of hypo/achlorhydric, which in normal individuals results in a reciprocal increase in gastrin release from the G cells of the gastric antrum causing hypergastrinemia[7,8,10,14,15]. This is most frequently due to chronic atrophic gastritis, commonly due to the presence of a *Helicobacter pylori* (*H. pylori*) infection that spares the antrum or due to pernicious anemia[7,8,10,14,15]. A second very frequent cause is the use of potent gastric acid anti-suppressant drugs such as proton-pump inhibitors (PPIs) (omeprazole, lansoprazole, pantoprazole, esomeprazole, rabeprazole), which is discussed in the next paragraph. Other less common causes of hypochlorhydric/achlorhydria include chronic renal failure and vagotomy, which can be distinguished by appropriate other laboratory/clinical investigations[5,8,10,13]. Similarly, the presence of gastric acid hypersecretion without hypergastrinemia can be seen in patients with idiopathic gastric acid hypersecretion and a few other uncommon conditions (mastocytosis, basophilic granulocytic leukemia)[8,16,17].

While the diagnosis of ZES sounds simple enough, in recent years it is becoming increasingly more difficult, due to a number of developments, and now, as pointed out in the above two papers, it is even further complicated. Let's first consider the issues that were complicating the diagnosis of ZES prior to these two papers. First, acid secretion is now rarely measured and therefore generally not available, so alternatives to the classical basal acid output assessment were proposed. These include, in addition to the presence of fasting hypergastrinemia, an endoscopic measurement of gastric acid output[18], the use of either pH paper or a pH meter to establish the presence of an acidic pH in gastric fluid, assessment for the presence of other features of ZES such as the presence of a tumor on imaging or pathologic studies and the development of gastric fluid pH criteria , that when coupled with the presence of hypergastrinemia, support the diagnosis of ZES[1,11,14]. Second, the widespread use of PPIs is markedly complicating the ability to diagnosis ZES, because their use interferes with both needed assessments to establish the diagnosis of ZES: the measurement of fasting gastrin levels and the assessment of acid secretion[5,10,12,14,19]. This occurs because PPIs have a very long duration of action, with their gastric acid suppressive action lasting up to one week[13,20,21] which not only contributes to their marked effectiveness, but also makes it difficult to withdraw patients from these drugs to assess gastric secretion[1,5,14]. Furthermore, their potent antisecretory activity can lead to fasting hypergastrinemia due to the PPIs and thus mislead one into suspecting ZES[14,19,22]. The result of the PPIs is that they both mask the diagnosis of ZES, leading to delays in diagnosis, because they effectively control all presenting clinical symptoms, but also they can lead to a false diagnosis[3,5,14,19,23]. False diagnoses occur because long-term use of PPIs can cause fasting hypergastrinemia in 80%-100% of patients without ZES[24-27] with fasting gastrin values > 4 fold increased in 20%-25% of patients[24-27] and in some cases the gastrin levels are > 10-fold elevated into ranges that are frequently thought to reflect ZES[25,26].

Commentary paper

In Poitras *et al*[1] two patients are reported in whom ZES was suspected (later proven) after presenting with severe symptoms of gastroesophageal disease, subsequently treated with PPIs with symptom improvement and when the PPIs were withdrawn, both patients developed severe complications of peptic disease (patient No. 1, esophageal stricture requiring repeated dilatations; patient No. 2, intestinal perforation). It was proposed that PPI therapy should always be maintained and diagnostic evaluations be performed while taking PPIs[1]. This is a novel recommendation and would have a marked effect on the diagnostic approach to patients suspected of having ZES. This recommended approach differs from the approach recommended in recent consensus guidelines and by most authorities in recent reviews, wherein, it is recommended that acid antisecretory drugs have to be stopped at some point to establish the diagnosis of ZES. Specifically, both the North American Neuroendocrine Tumor Network guidelines[9] and the European Neuroendocrine Tumor Network's guidelines[3,12], as well as a recent reviews of the diagnosis of ZES by a number of authorities[7,10,14,22,28], all recommend that PPIs need to be generally stopped to establish the diagnosis of ZES.

Is there any additional evidence to support this novel recommended diagnostic approach for ZES in paper 1[1]? Others have reported severe esophageal strictures in patients with ZES, since the time that antisecretory drugs were available, whose acid hypersecretion was not controlled[29-31] and in one large study[32], 8% (10/122) patients with ZES required repeated esophageal dilations, because of previous poor control of the acid hypersecretion. We don't find additional cases in the literature to patient No. 1 described in this report[1], however, we have seen one patient with ZES who developed a severe long, esophageal stricture requiring a stent, because antisecretory medications were withdrawn at an outside hos-

pital for diagnosis (unreported case). Similarly intestinal perforations have been reported in a number of patients with ZES since antisecretory drugs became available. Intestinal perforations were reported in 7% of patients with ZES in one large series (11/160 cases)[33] and in a number of other case reports[34,35], with the perforations occurring prior to the diagnosis in most cases. However, we have seen three cases of ZES patients who developed intestinal perforations when taken off of antisecretory drugs for diagnostic reasons[33,34] (unpublished 2 cases). One case occurred after a patient with suspected ZES reduced the PPI dose they were taking on their own because they developed constipation while taking the PPI and then presented with a duodenal perforation after 5 d of stopping the PPI in preparation for a secretin test[34]. Two other cases occurred prior to the use of PPIs early in our experience and we have seen no additional cases at National Institutes of Health (NIH) in the last 20 years in acid studies of more than 300 patients with ZES, the majority of who were taking PPIs prior to diagnosis. These latter results demonstrate that acid secretory studies can be safely carried out if proper precautions are taken, even if patients are taking PPIs, although it requires a center well versed in performing these studies, and a proven approach, one of which is discussed below, However, the query raised by Poitras *et al*[1] still remains, as to whether it is necessary to withdraw antisecretory medications in most patients with ZES to establish the diagnosis.

Unfortunately, the evidence suggests, as concluded in the accompanying editorial[14] to paper No. 1[1], that to clearly establish the diagnosis of ZES, some appropriate assessment of gastric acid acidity/secretion is required after withdraw of PPIs in almost every patient. What is the evidence? First, it is both fortunate and unfortunate that PPIs are so effective in patients with ZES as well as patients with idiopathic peptic disease. It is fortunate because PPIs are very effective at controlling gastric acid secretion in these patients[5,7,36,37]. However, it is unfortunate for diagnostic purposes, because their effectiveness results in that fact that with PPIs, the usual doses used in idiopathic peptic disease are often effective also in ZES and result in hypo/achlorhydria in both ZES patients and in patients without ZES. In contrast with Histamine H_2-receptor antagonists, frequently 10-fold greater doses than used in treating patients with idiopathic peptic disease are required in ZES patients, with more frequent dosing to control the hypersecretion, however these high doses in ZES and the usual doses in patients without ZES, rarely result in hypo/achlorhydria[5,7,36,37]. Therefore, in most patients taking PPIs where ZES is suspected, the gastric pH will not be < 2 (the range required for diagnosis of ZES)[7,11], and therefore physiological and pathological hypergastrinemia can not be distinguished on the drug. Second, there is no feature of the clinical course that unequivocally allows one to establish the presence of ZES, with most patients currently presenting with idiopathic peptic ulcer disease or gastroesophageal reflux disease which is indistinguishable from that seen in non-ZES patients[7,14,35,38]. Third, when ZES is suspected, a fasting gastrin level is almost invariably the first diagnostic study performed[7-10,12,14,39]. Unfortunately, there is no absolute level of fasting hypergastrinemia alone that can distinguish a patient with ZES from a patient without ZES[6,39]. This will be covered in more detail below in the discussion of Rehfeld *et al*[2], however a few additional points will be made here. In the most common cause of fasting hypergastrinemia, chronic atrophic gastritis, fasting gastrin levels > 70 fold elevated have been reported and levels > 1000 or > 2000 ng/L are not uncommon[40-43], which is a similar finding in patients with pernicious anemia[44]. These values overlap with 80%-100% of patients with ZES in various series[39]. Similarly, in patients taking PPIs without ZES, which is the also one of the most common causes of hypergastrinemia, the PPIs can lead to various degrees of hypergastrinemia in different patients. Although, as pointed out in a number of studies, PPIs frequently lead to < 3 fold increase in fasting gastrin and in some studies do not increase the value out of a normal range[22,45,46], this finding can not be relied on in an individual patient. This conclusion is firmly supported by various studies which report 80%-100% of the patients without ZES in their studies treated with PPIs develop hypergastrinemia, 20%-25% > 4 fold elevated, and values > 1000 pg/mL are not uncommon[24-27,47]. These levels of PPI induced increases in fasting gastrin overlap with that seen in more than 60% of patients with ZES[6,39,48,49]. Fourth, alone, no absolute level of any other tumor marker such as a serum chromogranin A (CgA) level, can establish the diagnosis of ZES. CgA levels are elevated in 90%-100% of patients with ZES, which can be contributed to by both the gastrinoma and the gastrin induced enterochromaffin-like (ECL) cell hyperplasia which is almost always present[50-55]. However, the main problem is that PPIs or high doses of other antisecretory drugs, can increase CgA levels in patients without ZES, within a few days of use, which is thought secondary to the PPI-induced hypergastrinemia causing gastric ECL proliferative effects[27,56,57]. The PPI induced increases in CgA can are usually < 4 fold, but can be up to 40-fold, which overlaps with values seen in > 90% of patients with ZES, as well as seen in numerous, non-ZES conditions[24,27,53,54,57-59]. Fifth, the recommended establishment of a diagnosis of ZES in a hypergastrinemic patient by other methods, as proposed in paper No. 1[1], such as by attempting to establish the presence of a neuroendocrine tumor (primarily by imaging studies) or by establishing the presence of a gastrinoma, is unlikely to be successful in many patients and may lead, in fact, to false diagnoses. It is likely to be unsuccessful in many patients because < 30% of patients with ZES at presentation at the current time, have liver metastases that possibly could be biopsied and the diagnosis of a gastrinoma established by immunohistochemistry[13,60,61]. Even this does not secure the di-

agnosis, because other pancreatic neuroendocrine tumors [non-ZES primitive neuroectodermal tumors (pNETs)] can stain occasionally positively for gastrin but not be associated with ZES or the portion of the neuroendocrine tumor biopsied may not show gastrin staining[62-69]. Furthermore, localization of a primary gastrinoma, even with the use of increasingly sensitive methods such as somatostatin receptor imaging or endoscopic ultrasound studies, misses most small duodenal tumors, which are present in 60%-80% of patients with ZES[70-75]. Furthermore, cross-sectional imaging studies (computed tomographic scanning, magnetic resonance imaging, trans-abdominal ultrasound) will be negative in > 60% of all patients with duodenal tumors and thus not be able to assist in suggesting the presence of a pNET in the majority of suspected cases, especially those patients being examined early in their course[71,72,76,77]. Lastly, sensitive methods such as somatostatin receptor scintigraphy can lead to false positive localization results and in one prospective study[78,79], 12% of all possible pancreatic endocrine tumor localizations were false positive. These include the presence of an accessory spleen, gallbladder retention, an abscess, various inflammatory processes, inadequate bowel cleansing, thyroid disease, various granulomatous lung diseases, and other neuroendocrine tumors/proliferations such as gastric ECL proliferation or other gastrointestinal-neuroendocrine tumors like gastric carcinoids tumors[78-81]. Therefore, one cannot conclude that localization on a somatostatin receptor scintigraphy study in a patient with hypergastrinemia necessarily equates to localization of a gastrinoma and establishment of the diagnosis of ZES. Fifth, besides the assessment of fasting gastrin levels and gastric fluid pH, various gastrin provocative tests (primarily after secretin, occasionally after glucagon)[14,48,49,82,83] are widely used in the diagnosis of ZES. The secretin test in particular has been well studied and in one recent detailed analysis of 537 patients with ZES in the literature as well as 293 NIH patients with ZES prospectively studied, the secretin test was shown to have a sensitivity of 94% with a specificity of 100% using a criterion of ⩾ 120 ng/L increase post secretin[49]. Couldn't the secretin provocative test be used while the patient is taking PPIs to circumvent the need to stop the PPI? Unfortunately, the answer is no, because a recent study[84] reports a false positive secretin test in a patient without ZES taking PPIs. Furthermore, another study demonstrated if patients are achlorhydric, false secretin positive tests can occur[85].

Commentary on paper

In the study by Rehfeld *et al*[2], Seven of 12 tested commercial kits inaccurately measure plasma concentrations of gastrin; these assays used antibodies with inappropriate specificity that were insufficiently validated. Misdiagnosis of gastrinoma based on lack of specificity of assays for gastrin results in ineffective or inappropriate therapy for patients with ZES.

Background: An accurate assessment of fasting serum gastrin levels (FSG) is central to the diagnosis of ZES and the diagnosis cannot be made without it. This is especially true, because this is the initial study that leads to the suspicion of ZES, in most cases[2,7,9,12,83]. An assessment of FSG is generally used as the initial study not only because of its convenience and widespread availability, but also because it is elevated in almost all patients with ZES, except for a few specific situations. In a review of 2229 cases of ZES from the literature[39], FSG levels were elevated in 97% of patients and in 309 patients with ZES seen at the NIH it was elevated in 99.3% of patients[39]. These results and others[6,86,87] demonstrate that normal FSG levels are uncommon overall in patients with ZES however, in a few small subgroups with active disease, normal values are not uncommon. This includes patients with active ZES who had had unsuccessful curative resection of a gastrinoma previously, who have ZES with multiple endocrine neoplasia type 1 (MEN1) post successful parathyroidectomy for hyperparathyroidism, in patients being treated with somatostatin analogues or occasionally in patients after various anti-tumor treatments (chemotherapy, chemo-embolization, *etc*.)[86-96]. Although many physicians think FSG levels are massively elevated in ZES and easy to distinguish from other disorders, unfortunately, as pointed out above, this is not the case. Chronic atrophic gastritis, other hypo/achlorhydric conditions, chronic renal failure and the use of PPIs can cause FSG levels that overlap with those seen in most patients with ZES. Furthermore, in 60%-65% of ZES patients when initially diagnosed, the FSG levels are < 10-fold elevated and these levels overlap with a number of other conditions, some of which are much more frequent than ZES, which can also be associated with gastric acid hypersecretion, such as seen in ZES[5,13,39,48,97]. This FSG level (i.e., > or < 10 × increased) is pointed out because the existing criteria used for the diagnosis of ZES in most guidelines and reviews is divided on the basis of the elevation of FSG[3,12,14,38,39]. If the FSG is > 10-fold elevated (usually > 1000 ng/L) and the gastric fluid pH < 2, a diagnosis of ZES is established after ruling out a possible retained antrum syndrome by history[12,14,38,39,98]. On the other had if the FSG is < 10-fold increased and the gastric fold pH < 2, other conditions need to be excluded including *H. pylori* infection, antral G cell syndromes, gastric outlet obstruction and renal failure[3,7,9,38,39]. In these cases a gastric analysis with determination of basal acid output, and a secretin provocative test is recommend, or if secretin is not available, a glucagon provocative test has been recommended[7,12,49,82,87]. These latter provocative tests involve the assessment of serum gastrin before and after a secretin or glucagon injection, so an accurate assessment of serum gastrin is essential to their correct interpretation.

Specific comments: Rehfeld *et al*[2] report that in many cases that the gastrin laboratory assays being used to assess FSG levels are not giving accurate results. Only 5 of the 12 commercial assays examined accurately assessed

FSG levels with the others giving FSG values either too high or too low and thus their results could lead to an over- or under-diagnosis of ZES, based on the FSG levels reported[2]. While gastrin provocative test results (secretin, glucagon) were not assessed, a similar result would be expected with these and thus the result would not be dependable in most cases. This report raises a number of problems for physicians trying to diagnose and treat patients with ZES. First, it demonstrates that FSG levels should not be compared from different laboratories using different assays unless some validation is performed. Second and more important, this report raises a real dilemma for the practicing clinician, because it raises the question of whether he can rely on FSG values reported to him by the laboratory he uses. There is no simple solution to this dilemma. The lists of laboratories assessed in this paper[2] can be consulted to see if the one used for the blood samples sent by for the clinician's patients(s) are on this list. Because the diagnosis of ZES has such significance for any patient and alternative approach as discussed in a section below is to refer the patient to center with known expertise, or to contact them and find out which laboratory in their area they recommend to assess FSG, and confirm the results using this laboratory.

Recommended approach to diagnosis of ZES (based on points raised in papers 1, 2)

First, it is essential to realize that patients with untreated gastric acid hypersecretion with ZES can develop complications rapidly and that this needs to be adequately treated before trying to establish a diagnosis, especially by stopping PPIs. There is no urgency in establishing the diagnosis. Therefore if the patient has active peptic ulcer disease or symptoms and the diagnosis of ZES is suspected, a FSG should be drawn and the acid hypersecretion adequately controlled [our initial starting dose is equivalent to omeprazole 60 *qd*[99,100], or if complicated disease (presence of MEN1, Billroth 2 surgery, or severe gastroesophageal reflux symptoms), we start with the equivalent of omeprazole 40 *bid*][37,101-103] and the patient should undergo an upper gastrointestinal endoscopy. We start with a higher PPI dose to make sure the acid hypersecretion is initially well controlled and then later it can be reduced in many patients[37,101]. During this endoscopy, gastric pH can be measured and also the size of gastric mucosal folds noted because 92% of ZES patients have prominent gastric folds[35]. Most patients can be satisfactorily treated by this PPI dose initially, however some require higher doses and therefore it is best to assess the control of acid hypersecretion on PPI[37,99,104], however, only a few specialty centers have this capability, and thus most use control of symptoms to monitor effectiveness of treatment. If the patient has symptoms of gastroesophageal peptic disease or active disease on endoscopy they should be treated for 8-12 wk until symptom free and then the upper gastrointestinal endoscopy repeated to make sure any peptic disease is resolved before attempting to establish the diagnosis by stopping PPIs. During this time the reliability of the FSG assay used needs to be explored both by reviewing the laboratories in paper No. 2[2] and contacting some group well versed in your area with the diagnosis of ZES that uses gastrin assays regularly. If the FSG is elevated it, of course this could be due to the PPI the patient was taking when the blood sample was drawn, which is the situation in most cases, so that the diagnosis remains unclear at this point, particularly if the gastric pH was > 2. At this point many authorities recommend that consideration be given to referring the patient to a group in your area well versed in the diagnosis of ZES[7,9,12,14]. If not possible, then after the repeat endoscopy shows healing of mucosal disease and the patient is asymptomatic, one can consider PPI withdrawal for diagnosis. Our approach is similar to outlined recently[14] and briefly, is to carefully consult with the patient about the need to keep in close contact, then to substitute an H_2 receptor antagonist (usually ranitidine 450-600 every 4 to 6 h) for the PPI for 3-5 d and then stop the ranitidine for 24 h allowing liberal use of antacids. Then on the day of the test we measure FSG × 2, and measure gastric pH and acid output. We usually perform a secretin test at the same time if our index of suspicion is high[49]. This circumvents the need to take the patient off of PPIs again at a later time if the secretin test is deemed necessary. If a gastric/esophageal pH probe is available the assessment of gastric pH and FSG at early times can be done with an attempt to document a pH < 2 with an elevated FSG.

One could ask at this point why go through all of this and why is it so important to establish the diagnosis of ZES correctly in most patients. The primary reason is that the diagnosis of ZES requires special treatment and the treatment must be continued life-long if the patient is not cured[7,9,105]. If the patient is not cured, life-long PPI treatment will be required, the doses may be different than that usually used in patients with idiopathic peptic disease, periodic assessment for the presence of MEN1 will be needed because 20%-30% of ZES patients have this and its diagnosis may not be initially evident and treatment directed at the gastrinoma, which are malignant in 60%-90% of cases, must be considered[13,35,38,106,107]. The latter include periodic assessment of tumor location/extent with imaging studies, consideration of surgical resection which is recommended in ZES patients in whom MEN1 is not present, life expectancy is good and no serious surgical contra-indications are present[7,71,108-110]. Therefore a diagnosis of ZES markedly effects clinical management and thus needs to be well established.

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GUIDELINES FOR BASIC SCIENCE

Molecular targets in the treatment of alcoholic hepatitis

Ashwin D Dhanda, Richard WL Lee, Peter L Collins, C Anne McCune

Ashwin D Dhanda, Peter L Collins, C Anne McCune, Department of Liver Medicine, University Hospitals Bristol NHS Foundation Trust, BS2 8HW Bristol, United Kingdom
Ashwin D Dhanda, Richard WL Lee, Department of Cellular and Molecular Medicine, School of Clinical Sciences, University of Bristol, BS9 1TD Bristol, United Kingdom
Author contributions: Dhanda AD wrote the initial draft; Lee RWL, Collins PL and McCune CA reviewed and edited the manuscript.
Correspondence to: Dr. C Anne McCune, MD, FRCP, Consultant Hepatologist, Department of Liver Medicine, University Hospitals Bristol NHS Foundation Trust, Bristol Royal Infirmary, Marlborough Street, BS2 8HW Bristol, United Kingdom. anne.mccune@uhbristol.nhs.uk
Telephone: +44-117-3422432 Fax: +44-117-3423533
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Abstract

Alcohol related costs to health and society are high. One of the most serious complications of alcohol misuse to the individual is the development of alcoholic hepatitis (AH), a clinical syndrome of jaundice and progressive inflammatory liver injury in patients with a history of recent heavy alcohol use. It has a poor outcome and few existing successful therapies. The use of glucocorticoids in patients with severe AH is still controversial and there remains a group of patients with glucocorticoid-resistant disease. However, as our understanding of the pathogenesis of the condition improves there are opportunities to develop new targeted therapies with specific actions to control liver inflammation without having a detrimental effect on the immune system as a whole. In this article we review the molecular mechanisms of AH concentrating on the activation of the innate and adaptive immune response. We consider existing treatments including glucocorticoids, anti-tumor necrosis factor therapy and pentoxifylline and their limitations. Using our knowledge of the disease pathogenesis we discuss possible novel therapeutic approaches. New targets include pro-inflammatory cytokines such as interleukin (IL)-17, chemokines and their receptors (for example IL-8, CXCL9 and CXCR3) and augmentation of anti-inflammatory molecules such as IL-10 and IL-22. And there is also future potential to consider combination therapy to selectively modulate the immune response and gain control of disease.

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Key words: Alcoholic hepatitis; Tumour necrosis factor-α; Pentoxifylline; Interleukins; Chemokine receptors

Peer reviewers: Dr. Philip Abraham, Professor, Consultant Gastroenterologist and Hepatologist, P. D. Hinduja National Hospital and Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400 016, India; Juan-Ramón Larrubia, PhD, Gastroenterology Unit and Liver Research Unit, Guadalajara University Hospital, Donante de Sangre s/n, 19002 Guadalajara, Spain; Luis Bujanda, PhD, Professor, Departament of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain

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INTRODUCTION

Alcohol related morbidity in developed countries is second only to tobacco use and is responsible for 2.5 million deaths globally each year[1]. It costs 1% of the GDP of middle to high income countries[2]. In the United Kingdom, deaths from alcohol related liver disease have increased by 36% between 2001 and 2008[3]. Alcoholic hepatitis (AH) is a clinical syndrome characterised by jaundice and progressive inflammatory liver injury in patients with a history of recent heavy alcohol misuse. Severe cases are associated with a high mortality of around 30%-50% at 28 d[4,5]. However, the pathogenesis of the

condition is incompletely understood and there is a lack of targeted therapy.

It is clear that alcohol acts both directly on the liver, causing cell death by toxic mediators, and *via* activation of the immune system. Over the past 2 decades considerable headway has been made into understanding the immune basis of the disease with studies on pro- and anti-inflammatory cytokines, cell trafficking to the liver and mechanisms of hepatocyte death. This work has been hampered by the absence of a suitable animal model of the disease and much of the relevant work has been inferred from other models of acute hepatic injury or through chronic ethanol feeding. Although these models share several important similarities with human AH, such as neutrophil accumulation in the hepatic ischaemia model or hepatocyte ballooning in the ethanol-fed rat model, they still have significant differences. Neither the acute hepatic ischaemia model nor the carbon tetrachloride model involves alcohol metabolism and alcohol related liver damage. The chronic ethanol feeding model does not mirror the acute phase of inflammation in AH and instead is more similar to alcoholic steatohepatitis[6]. Therefore, at present we must interpret results from these studies with caution and focus attention on studies on human tissue.

In this review, we consider the pathogenesis of AH, focussing on the contribution of immunity (Figure 1), and relate this to the use of targeted molecular therapies. We briefly discuss the evidence for anti- tumor necrosis factor-α (TNFα) therapies and pentoxifylline, both of which have been extensively studied in AH, but do not intend this to be a definitive systematic review. For further discussion on these 2 treatments we refer the readers to recent comprehensive reviews[7-9]. We have emphasised the molecules that we believe have the greatest future therapeutic potential.

PATHOGENESIS

Within the liver alcohol can be metabolised to acetaldehyde by three enzyme systems: alcohol dehydrogenase in the cytosol, the microsomal ethanol-oxidising system and peroxisomal catalase. These metabolic pathways lead to the production of reactive oxygen species and cause lipid peroxidation. There is a reduction in the hepatoprotective methyl donor S-adenosylmethionine and mitochondrial glutathione. Acetaldehyde forms adducts with proteins, lipids and DNA, impairing their function and promoting DNA damage (see Setshedi *et al*[10] for a detailed review on this subject). However, it is not only the direct toxic effect of alcohol on the liver that causes damage but also activation of the immune response.

Activation of the innate immune system

Alcohol is known to change the gut microbiota[11] and can increase numbers of both aerobic and anaerobic organisms[12]. In addition, metabolism of alcohol by gut bacteria results in production of acetaldehyde which has a direct effect on the gut epithelium, opening up tight junctions, increasing its permeability[13,14]. This allows the presentation of bacteria or microbial cellular components to cells of the innate immune system. These pathogen-associated molecular patterns are recognised by toll-like receptors (TLRs) which are expressed on a wide variety of immune and stromal cells, in particular on monocytes and macrophages. Gut-derived endotoxin [or lipopolysaccharide (LPS)] crosses into the portal venous system and activates TLR4 on $CD14^+$ cells. Higher levels of LPS have been demonstrated in the portal veins of patients with alcohol related cirrhosis compared to non-alcohol related cirrhosis controls[15]. *Via* adaptor molecules MyD88 and toll/interleukin (IL)-1 receptor domain-containing adaptor inducing interferon-β, the signalling pathway converges on a few transcription regulators [nuclear factor κB (NF-κB), activator protein 1 (AP-1) and interferon regulatory factors], which lead to a pro-inflammatory immune response. Signalling through TLR4 plays an important role in the development of alcohol related liver damage as evidenced by TLR-mutant mice which are resistant to alcohol and have lower levels of circulating TNFα despite having higher circulating LPS[16]. Modulation of the gut microbiota with pro- or antibiotics reduces circulating levels of LPS in mouse models[17,18]. Activation of TLR4 on macrophages and kupffer cells (liver resident macrophages) leads to secretion of pro-inflammatory cytokines such as IL-1, IL-6 and TNFα, which act on surrounding hepatocytes and stromal cells as well as activating the adaptive immune response.

Activation of the adaptive immune system

It is clear that T-lymphocytes play a role in the development of AH with increased levels of $CD4^+$ and $CD8^+$ T-lymphocytes found in liver biopsy material from patients with AH compared to healthy controls[19]. The liver has a unique dual blood supply with both portal venous and hepatic arterial blood draining into the hepatic sinusoids. Hepatic sinusoid endothelial cells (HSEC) are therefore exposed to large numbers of leukocytes and HSEC expression of adhesion molecules and chemokine receptors enables adherence of lymphocytes and transmigration into the hepatic parenchyma to the site of inflammation. Unlike other endothelial cells HSEC express low levels of selectins[20] but high levels of intercellular adhesion molecule-1 (ICAM-1), vascular adhesion protein-1 (VAP-1) and vascular cell adhesion molecule-1 (VCAM-1)[21]. Inhibition of ICAM-1 and VCAM-1 reduce lymphocyte adhesion in *in vitro* flow-based adhesion experiments[21].

Liver infiltrating lymphocytes express high levels of CXCR3 and migrate towards its ligands CXCL9 and CXCL10[22], which are produced by a variety of liver cells including hepatic stellate cells and activated myofibroblasts[23]. High levels of TNFα found in AH stimulate production of CXCL10 from hepatocytes and HSECs increasing T cell recruitment to the liver[24]. Data from

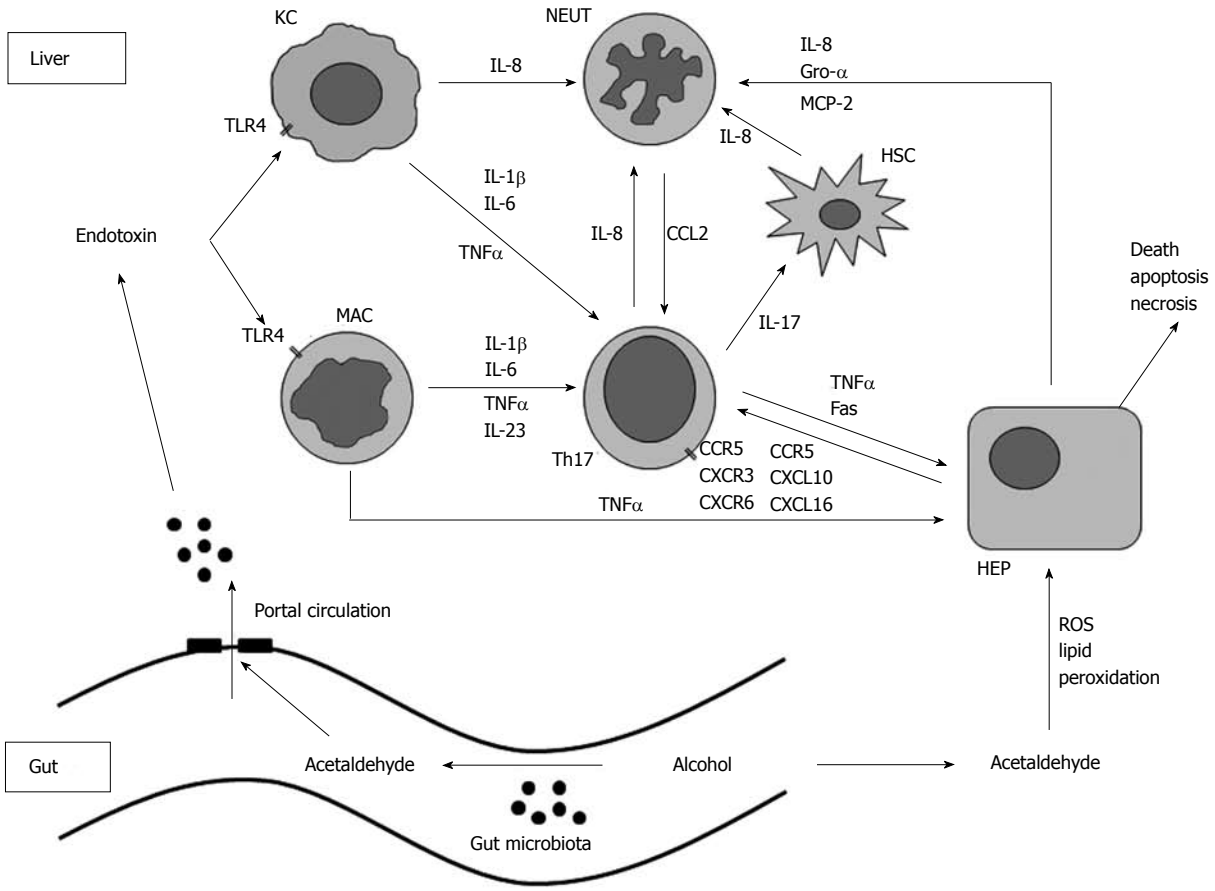


Figure 1 The effects of alcohol on the innate and adaptive immune system. Alcohol is metabolised by gut microbiota to acetaldehyde which has a direct effect on the epithelial tight junctions, making them more leaky. Endotoxin and other pathogen-associated molecular patterns enter the portal circulation and act *via* toll-like receptor (TLR) 4 receptors on kupffer cells (KCs) and macrophages (MAC), activating them to produce pro-inflammatory cytokines and chemokines, which act on hepatocytes, neutrophils (NEUT) and T cells. Both $CD4^+$ and $CD8^+$ T cells are recruited into the liver but there is a significant proportion of interleukin (IL)-17 secreting $CD4^+$ Th17 cells, which in turn activate stellate cells to produce IL-8. T cells also act directly on hepatocytes *via* the tumor necrosis factor (TNF) receptor 1 or Fas pathways leading to apoptosis. Alcohol also has a direct effect on hepatocytes (HEP) through metabolism by alcohol dehydrogenase, the microsomal ethanol-oxidising system and peroxisomal catalase to acetaldehyde, which damages cells by the formation of reactive oxygen species (ROS) and increased lipid peroxidation. HSC: Hepatic stellate cell; MCP: Monocyte chemotactic protein.

patients with chronic hepatitis C infection also demonstrate that liver infiltrating lymphocytes express high levels of chemokine receptor CXCR3 as well as others such as CCR5 and CXCR6[24,25].

Once T-lymphocytes have been recruited to the site of inflammation there is evidence that the $CD4^+$ subset mediates hepatocyte death. Adoptively transferred liver associated $CD4^+$ T cells from ethanol-fed rats demonstrated greater hepatocyte apoptosis in recipient rats compared to those transferred from non-ethanol-fed rats[26].

Hepatocyte death can occur by necrosis or apoptosis. Necrosis is characterised by ballooning and is clearly described in cases of AH. However, apoptosis has also been identified as an important mechanism of AH. There is greater hepatocyte apoptosis in the livers of patients with AH compared to controls[27] and the degree of apoptosis correlates with clinical measures of severity[28]. Apoptosis may be caused by direct cytotoxic T cell activation of the Fas apoptosis pathway, which in turn can induce IL-8, a neutrophil chemotaxin. Alternatively, TNFα binding to TNF-R1, a member of the death receptor family, can initiate apoptosis. As discussed above, TNFα is significantly elevated in severe AH suggesting it can mediate hepatocyte death by this mechanism as well as by direct toxic effects leading to necrosis.

Neutrophil recruitment

The degree of intrahepatic neutrophil infiltration correlates with severity of AH[29] although their exact role in the pathogenesis of the disease is less well understood. Data from animal models of other acute liver injury such as ischaemia suggest that neutrophils are activated by cytokines including TNFα, IL-1β and IL-17 and are recruited to the hepatic parenchyma by CXC chemokines such as Gro-α[29], IL-8 and monocyte chemoattractant protein 2[30]. CXC chemokines are particularly important for neutrophil infiltration in AH[31]. These neutrophils migrate into the parenchyma and cause hepatocyte necrosis by release of reactive oxygen species and proteases[32].

With this understanding of the molecular mechanisms of the pathogenesis of AH, we can more effectively target therapy.

Existing therapy for severe AH

Glucocorticoids: Glucocorticoid therapy was first demonstrated to be beneficial in the treatment of patients with severe AH in 1978[4]. Glucocorticoids are potent immunosuppressants which have wide ranging effects on a large number of cell types. Key among their actions is suppression of the pro-inflammatory transcription factors NF-κB and AP-1[33] resulting in lower levels of circulating TNFα and IL-8[34].

Glucocorticoid treatment has been evaluated in many clinical trials and debate continues whether they are beneficial with conflicting results of meta-analysis[35-37]. The largest placebo-controlled trial recruited a heterogeneous group of 90 patients (including those with moderate and severe disease as well as end stage alcoholic liver disease) and failed to show a survival benefit at 30 d[38]. Another trial recruiting only patients with histologically proven AH showed a survival benefit with glucocorticoid treatment at 2 mo[39] but no difference at long term follow-up at 2 years[40]. More recent meta-analysis of individual patient data demonstrated a survival benefit with glucocorticoid treatment in patients with severe AH[5]. However, they identify a proportion of glucocorticoid non-responders, with a significantly worse outcome, by applying the Lille model[41] at day 7 of treatment. This highlights the fact that as in other inflammatory diseases such as asthma and rheumatoid arthritis there is a proportion of patients resistant to steroid treatment[42]. In addition, the non-specific effect of glucocorticoids means that there is a risk of infective complications. Other therapeutic options are therefore essential.

Pentoxifylline: Pentoxifylline, a phosphodiesterase inhibitor, has a moderate effect on TNFα levels[43-45] although circulating TNFα levels in patients on active treatment did not show any difference from those on placebo in a randomised controlled trial[46]. The effects of pentoxifylline are likely to be less specific than inhibition of a single cytokine. In animal models of liver injury it has shown a reduction in pro-fibrogenic cytokines such as pro-collagen I and transforming growth factor beta 1[47]. Although the exact mechanism of action of pentoxifylline in AH is not clear several trials have shown a survival benefit, predominantly through a significant reduction in mortality due to hepatorenal syndrome[46,48]. However, a recent Cochrane systematic review was unable to draw firm conclusions about its beneficial effect due to probable bias in the design of a number of trials[49]. A large United Kingdom multi-centre randomised placebo-controlled trial of pentoxifylline, prednisolone or combination is currently underway, which hopes to provide a definitive answer as to whether these treatments are beneficial in patients with severe AH.

Anti-oxidant therapy: Oxidative stress plays an important role in the pathogenesis of AH (Dey *et al*[50]). In an attempt to reduce its toxic effects, several clinical trials of anti-oxidant agents have been undertaken either in combination with glucocorticoids or as a monotherapy. A recent randomized controlled trial (RCT) of 5 d of intravenous N-acetylcysteine (NAC) or placebo with 4 wk of glucocorticoids[51] demonstrated a reduction in mortality at 1 mo in the NAC arm but failed to reach significance at 2 mo and 6 mo (the primary outcome of the trial). A lower incidence of infections was noted in the NAC treated group.

A further RCT has been reported using an anti-oxidant cocktail (including NAC and vitamin E) with or without concomitant glucocorticoids, which failed to show an improvement in 6 mo survival[52]. A different anti-oxidant cocktail was tested against glucocorticoid therapy but a higher number of deaths at 30 d was reported in the anti-oxidant treatment arm[53]. Vitamin E versus placebo also failed to demonstrate any benefit in outcome[54].

Anti-TNFα therapy: Anti-TNF therapy with chimeric monoclonal antibody (infliximab) or fusion proteins (etanercept) have proved successful in other inflammatory conditions such as rheumatoid arthritis[55] and Crohn's disease[56]. With high levels of TNFα demonstrated in patients with severe AH[57] and since TNFα predicts short-[58] and long-term survival[59], it is an appropriate target for effective therapy.

In ethanol-fed rats treated with anti-TNF antibody, there was a significant improvement in hepatic inflammation, although serum levels of TNFα remained unchanged[60] supporting a role for this drug in human AH. An open label pilot study of a single dose of 5 mg/kg infliximab in combination with glucocorticoids found a significant reduction in Maddrey Discriminant Function[61]. An RCT of 3 infusions of infliximab 10 mg/kg or placebo at weeks 0, 2 and 4 in combination with prednisolone was stopped early due an excess of deaths in the active treatment group due to infection[62]. An RCT of etanercept also found that there was a significantly poorer outcome for patients on active treatment due to infection[63]. Lower doses of infliximab or less aggressive loading regimes may reduce the incidence of infection. Alternatively, combination therapy with an anti-inflammatory treatment such as recombinant IL-10 or IL-22 (see below) may boost the immune response to infections.

Alteration of the gut microbiota

Alcohol alters the gut microbiota and together with increased intestinal permeability there is activation of the innate immune system to produce an inflammatory response in AH. No clinical trial of probiotics has yet been reported in patients with AH. However, there is evidence that changing the bowel microbiota with probiotics in patients with alcohol related cirrhosis reduces the production of pro-inflammatory cytokines[64] and improves liver function tests in patients with alcoholic psychosis[65]. Further investigation is required to determine whether probiotic therapy is beneficial in AH. Alteration of the gut microbiota with non-absorbable antibiotics

such as rifaximin may also be beneficial and has recently been proven effective for the prevention of hepatic encephalopathy in patients with cirrhosis[66].

The treatments for AH discussed so far have wide-ranging effects on the immune system. These non-targeted therapies have failed to demonstrate a clear benefit in outcome. Both anti-TNFα therapy and glucocorticoids suppress the appropriate immune response to infection, resulting in a higher incidence of serious infections. Over the last decade the emphasis in treatment of inflammatory conditions has shifted to a more targeted approach to lessen the impact of inflammatory mediators, while reducing the systemic complications of treatment.

Future molecular targets

IL-10: IL-10 is a potent anti-inflammatory cytokine, suppressing the production of inflammatory cytokines (including TNFα) by T helper cells, monocytes and kupffer cells. IL-10 can also inhibit production of reactive oxygen species by neutrophils[67]. IL-10 is upregulated in surviving patients that had a response to glucocorticoid therapy[34]. This is consistent with studies of glucocorticoid effects on T cells in other inflammatory conditions such as asthma[68]. Therefore increasing the levels of IL-10 in patients with significant inflammation may prove to be a useful therapeutic target.

Unfortunately, treatment with recombinant human IL-10 (rhuIL-10) in patients with Crohn's disease has proved disappointing. Although well tolerated, it failed to induce clinical remission or response in patients with mild to moderately active Crohn's disease[69,70] and a recent systematic review suggested a lack of effect of rhuIL-10 in patients with Crohn's[71]. One study found that administration of rhuIL-10 lead to an increased capacity of leucocytes to produce interferon-gamma (IFNγ), a potent Th1 secreted pro-inflammatory cytokine[72], which may explain its lack of anti-inflammatory effect.

In the context of liver disease, rhuIL-10 has been trialled in chronic hepatitis C. Thirty patients with advanced fibrosis who failed treatment with antiviral therapy were treated with rhuIL-10 administered subcutaneously daily or 3 times weekly for 1 year[73]. There was a significant improvement in serum transaminase and a reduction in inflammation scores in 13 of 28 patients and fibrosis scores in 11 of 28 patients and a 2 fold reduction in the number of hepatitis C virus (HCV) specific $CD4^+$ IFNγ secreting T cells. However, there was a significant increase in HCV RNA levels which returned to baseline 6 mo after end of treatment.

A pilot open label study of rhuIL-10 in combination with glucocorticoids in 8 patients with severe AH failed to show any changes to neutrophil-derived or serum IL-8 and TNFα production[74]. No significant differences in mortality or MDF were observed in comparison to the control group. This may be explained by the fact that although in patients with severe AH IL-10 is upregulated, it is not sufficient to reduce the levels of TNFα[75]. Therefore a combination of cytokine targets to inhibit TNFα while augmenting IL-10 may be a useful approach.

IL-22, a member of the IL-10 family, shares its anti-inflammatory effects and potentiation of this molecule may be more beneficial than the use of rhuIL-10 since the IL-22 receptor is only expressed on epithelial cells such as hepatocytes. A study in a chronic ethanol-fed mouse model showed that treatment with recombinant IL-22 ameliorates liver injury and hepatic oxidative stress[76]. In a murine model of acute hepatitis IL-22 receptor is upregulated on hepatocytes and blockade of IL-22 exacerbates disease while administration of IL-22 ameliorates it[77]. Interestingly IL-22 is produced by Th17 cells, thought to be pathogenic (see below). Perhaps the strategy of blocking IL-17 in conjunction with augmenting IL-22 will have a more synergistic effect.

IL-8: Inhibition of neutrophil mediated hepatic damage may also prove to be an important therapy. Levels of *IL-8* gene expression and serum protein are elevated in patients with AH and higher levels are associated with a poorer outcome[31]. *IL-8* gene expression is also related to neutrophil hepatic infiltration as well as increased portal pressure[31]. IL-8 antagonism may reduce neutrophil recruitment into the liver without impairing the neutrophils' function in host defence and elimination of pathogens.

Hepatocyte growth factor: Hepatocyte growth factor (HGF) is thought to play an important role in the regeneration of the liver after an injury such as AH. It reduces hepatocyte apoptosis[78] as well as reducing ethanol-related oxidative damage[79]. Higher levels of HGF correlate with greater hepatocyte proliferation in AH[80] and are associated with better outcome[81]. Treatment of alcohol fed mice with recombinant HGF reduced the development of fatty liver[82]. Interestingly, HGF is produced by neutrophils and the degree of neutrophil infiltration in the liver correlates with levels of HGF[83]. Therefore a strategy of blocking neutrophil trafficking to the liver by IL-8 antagonism may also have a detrimental effect by reducing the liver's regenerative capacity. Combination of IL-8 antibodies with recombinant HGF or *HGF* gene therapy may reduce the inflammatory effects of neutrophils while maintaining the beneficial effects on hepatocyte proliferation.

T cells: T cells play a role in the recruitment of neutrophils and the perpetuation of inflammation in AH. Reduction in T cell proliferation through targeting IL-2, a T cell proliferation and survival factor, may reduce the number of pro-inflammatory cells within the inflamed liver. Inhibition of IL-2 receptor alpha (CD25) with a chimeric monoclonal antibody reverses glucocorticoid resistance *in vitro* in peripheral blood mononuclear cells from patients with AH[42]. This monoclonal antibody (basiliximab) is licensed for use in acute cellular rejection of cadaveric renal transplants. Promising results

Table 1 Summary of mechanism of action and existing evidence about potential future molecular targets for the treatment of severe alcoholic hepatitis

Treatment	Mechanism of action	Clinical evidence
rhu IL-10	Suppression of pro-inflammatory cytokines, e.g., TNFα Suppression of reactive oxygen species	Phase 2 study in severe AH with concomitant glucocorticoids showed no significant difference in mortality or levels of IL-8 and TNFα[74]
rhu IL-22	Increased levels of anti-inflammatory IL-22 Reduced oxidative stress	Reduced liver injury in ethanol-fed rat model[76] and acute hepatitis mouse model[77]. No studies in human liver disease yet reported
Anti-IL-8 antibody	Reduced neutrophil recruitment to liver	Phase 2 studies in COPD demonstrated reduced dyspnea[103]. No studies in human liver disease have yet been reported
rhu hepatocyte growth factor	Reduced hepatocyte apoptosis	HGF levels correlate with outcome in severe AH[81]. Well tolerated in a small phase 1/2 trial in fulminant hepatitis but no clear clinical benefit was seen[104]
Basiliximab (anti-CD25 antibody)	Reduced T cell proliferation and subsequent pro-inflammatory cytokine production	Reverses *in vitro* glucocorticoid resistance in patients with severe AH[42]. No clinical trials yet conducted in liver disease
Secukinumab (anti-IL-17A antibody)	Reduced pro-inflammatory IL-17A	Well tolerated in patients with rheumatoid arthritis, psoriasis or uveitis[93]. No clinical benefit in crohn's disease[94]. No clinical trial yet reported in liver disease
Anti-CXCL10 antibody	Reduced Th1 cell recruitment to liver	CXCR3 (receptor for CXCL10) is elevated in patients with chronic hepatitis C infection[95]. Phase 2 clinical trial is underway in patients with PBC

rhu: Recombinant human; AH: Alcoholic hepatitis; TNFα: Tumor necrosis factor-α; HGF: Hepatocyte growth factor; PBC: Primary biliary cirrhosis; IL: Interleukin; COPD: Chronic obstructive pulmonary disease.

from an open label study in patients with glucocorticoid resistant ulcerative colitis showed a benefit[84] but has not been borne out in a double-blind randomised controlled trial[85]. The immunogenicity of the drug together with a high achievement of clinical response and remission in the placebo arm, may have contributed to the lack of benefit over placebo in this study. However, given in a single dose (shown to be less immunogenic in renal transplant patients[86]) basiliximab may prove to be a useful adjunct to glucocorticoid therapy in patients who do not respond to this therapy.

Th17 cells: Recently, T cells producing IL-17 (known as Th17 cells) have been ascribed a central role in the pathogenesis of many inflammatory and autoimmune conditions[87] and are present in chronically inflamed tissues[88]. The generation of Th17 cells in humans requires IL-1β and IL-6 with IL-23 as a survival factor[89]. IL-17 can itself act as a neutrophil chemotaxin but can also stimulate production of other chemotaxins such as IL-8 and CXCL1[90]. To date, 6 Th17 cytokines have been described (known as IL-17A-F)[91], the most studied being IL-17A.

Serum levels of IL-17 are higher in patients with AH compared to healthy and HCV controls[92]. From the same study liver biopsy material from patients with AH showed significantly higher numbers of infiltrating IL-17$^+$ cells compared with alcohol related cirrhosis samples and the number of infiltrating cells correlated with the Maddrey Discriminant Function. The increased levels of IL-17 within the liver are likely to act on hepatic stellate cells, which when stimulated with IL-17 increase chemotaxis of neutrophils[92].

Phase 1 trials of humanised anti-IL-17A monoclonal antibody (secukinumab) have proved successful in the treatment of rheumatoid arthritis, psoriasis and uveitis[93] with phase 2 and 3 trials in rheumatoid arthritis ongoing. Disappointingly, small benefit was seen over placebo in patients with active Crohn's disease[94], which may be due to low concentration of the drug at the site of inflammation or presence of other Th17 produced cytokines such as IL-17F. To date no studies of secukinumab have been reported in patients with liver disease.

Lymphocyte trafficking to the liver

A number of chemokines and receptors have been identified as playing a role in neutrophil, macrophage or lymphocyte trafficking to the liver. Targeting these receptors or blocking their ligands could limit liver infiltration and subsequent damage leaving cells to perform other functions such as clearance of infection in the periphery. Several possible targets are appealing: CXCR3 and its ligand CXCL10 appears to be important in liver disease. CXCR3 is expressed on Th1 cells and intrahepatic CXCR3 is upregulated in patients with chronic hepatitis C infection[24,95]. Although no studies have yet been performed in AH, a monoclonal antibody to CXCL10 is currently under evaluation in patients with primary biliary cirrhosis, Crohn's disease and ulcerative colitis. However, this approach may have its limitations; a murine model of IFNγ mediated hepatitis [by Concanavalin A (ConA) challenge] demonstrated that CXCR3$^{-/-}$ mice developed a more severe hepatitis and failed to induce tolerance *via* conversion to an IL-10 secreting regulatory T cell (Treg) phenotype on re-challenge with ConA[96]. This suggests that CXCR3 is required for liver homing of lymphocytes in inflammation with subsequent development of an anti-inflammatory IL-10 secreting

Treg phenotype to limit hepatic inflammation. Therefore inhibition of the initial accumulation of T cells may lead to alteration of hepatoprotective downstream events. Combination therapy with anti-CXCL10 and rhuIL-10 or IL-22 may be an option to maintain the protective effects of IL-10 secreting Tregs.

VAP-1 is another potential target; VAP-1 is elevated in patients with inflammatory liver conditions[97] but also correlates with disease severity in AH[98]. Blockade of this molecule reduces peripheral blood and liver derived lymphocyte migration across HSECs[99].

Selective inhibition of T cell subsets, such as Th17 cells, from infiltrating the liver may reduce the most pro-inflammatory cell population from reaching the liver and causing more damage. However, the migratory fate of Th17 cells in humans is still poorly understood. Two chemokines, CCR4 and CCR6, have been identified as playing a role of Th17 migration in inflammatory disease[100,101] but these are also expressed on a variety of other T cell subsets[102]. Ongoing work is hoping to elucidate this pathway.

In conclusion, broad spectrum therapy for the treatment of severe AH has not been demonstrated to be clearly beneficial mainly due to development of infective complications. With our increasing understanding of the pathophysiology of the disease together with its immunological mechanisms, we have the opportunity to develop targeted molecular approaches (Table 1). However, work on AH remains challenging in the absence of an appropriate animal model. Many of the pre-clinical studies use chronic ethanol feeding murine models or we must infer results from non-alcoholic models of acute liver injury such as ischaemic or carbon tetrachloride induced hepatitis. This deficit must be addressed so we can further our understanding of the pathogenesis of this disease. Furthermore, clinical studies in AH lag far behind other inflammatory and autoimmune conditions such as rheumatoid arthritis and Crohn's disease. Learning from these studies we have seen that selected cytokine inhibition has rarely been successful (with TNFα being a notable exception). We therefore would strongly endorse future assessment of combination therapy such as recombinant IL-10 or IL-22 with anti-IL-17, HGF with anti-IL-8, glucocorticoids with IL-10, IL-22 or anti-CD25 or selective inhibition of chemokines and cytokines within the liver.

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GUIDELINES FOR CLINICAL PRACTICE

Checkmate to liver biopsy in chronic hepatitis C?

Anca Trifan, Carol Stanciu

Anca Trifan, Carol Stanciu, "Gr. T. Popa" University of Medicine and Pharmacy, "St. Spiridon" University Hospital, Independenţei 1, 700111 Iaşi, Romania
Author contributions: Trifan A and Stanciu C equally participated in the conception, design and drafting of this article; both authors revised the article critically for important intellectual content and gave the final approval for the version to be published.
Correspondence to: Carol Stanciu, MD, FRCP, Gastroenterology and Hepatology Center, "Sf. Spiridon" University Hospital, Independenţei 1, 700111 Iaşi,
Romania. stanciucarol@yahoo.com
Telephone: +40-72-2306020 Fax: +40-23-2264411
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Abstract

Liver biopsy (LB) has traditionally been considered the gold standard for pretreatment evaluation of liver fibrosis in patients with chronic hepatitis C (CHC). However, LB is an invasive procedure with several shortcomings (intra- and interobserver variability of histopathological interpretation, sampling errors, high cost) and the risk of rare but potentially life-threatening complications. In addition, LB is poorly accepted by patients and it is not suitable for repeated evaluation. Furthermore, the prevalence of CHC makes LB unrealistic to be performed in all patients with this disease who are candidates for antiviral therapy. The above-mentioned drawbacks of LB have led to the development of noninvasive methods for the assessment of liver fibrosis. Several noninvasive methods, ranging from serum marker assays to advanced imaging techniques, have proved to be excellent tools for the evaluation of liver fibrosis in patients with CHC, whereas the value of LB as a gold standard for staging fibrosis prior to antiviral therapy has become questionable for clinicians. Despite significant resistance from those in favor of LB, noninvasive methods for pretreatment assessment of liver fibrosis in patients with CHC have become part of routine clinical practice. With protease inhibitors-based triple therapy already available and substantial improvement in sustained virological response, the time has come to move forward to noninvasiveness, with no risks for the patient and, thus, no need for LB in the assessment of liver fibrosis in the decision making for antiviral therapy in CHC.

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Key words: Liver biopsy; Fibrosis; Noninvasive methods

Peer reviewers: Giuseppe Montalto, Professor, Clinical Medicine and Emerging Diseases, University of Palermo, via del Vespro, 141, 90100 Palermo, Italy; Masahito Uemura, MD, Associate Professor, Third Department of Internal Medicine, Nara Medical University, Shijo-cho, 840, Kashihara, Nara 634-8522, Japan; Dr. Bernardo Frider, MD, Professor, Department of Hepatology, Hospital General de Agudos Cosme Argerich, Alte Brown 240, Buenos Aires 1155, Argentina

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INTRODUCTION

Chronic hepatitis C (CHC) is a major public health concern, with around 180 million individuals affected worldwide[1]. Liver fibrosis and its end-point cirrhosis are the main causes of morbidity and mortality in patients with CHC[2]. Information on the stage of liver fibrosis is useful in patients with CHC not only for estimation of prognosis, but also for indication of antiviral therapy. Early international guidelines, consensus statements and expert panel opinions on the management of CHC unanimously recommended that decisions on treatment should be made only after performing a liver biopsy (LB) for pretreatment evaluation of the disease[3-5]. Consequently, antiviral treatment for patients with CHC has been indicated only for those with moderate to severe

stages of fibrosis (Metavir F2, F3 or F4), while patients with no or minimal fibrosis (Metavir F0, F1) have not been treated[6]. The rationale of such a strategy was to treat all patients with advanced fibrosis to halt disease progression and prevent complications, rather than those with no or minimal fibrosis who may await better treatments considering the slowly progressing natural history of CHC[7]. The recommendations mentioned above led to the routine performance of LB in nearly all patients who were newly diagnosed with CHC and potential candidates for antiviral therapy. More recent guidelines[8] still recommend LB in making treatment decisions, although it has been recognized that it is not necessary in patients with genotype 2 or 3, who can have as high as a 80% sustained virological response (SVR) rate.

For several decades, LB has been widely regarded as the gold standard for the staging of liver fibrosis[9]. However, LB is an invasive procedure and it is sometimes associated with rare but severe complications[10]. In addition, LB has several drawbacks (intra- and interobserver variability in histopathological interpretation, sampling errors, variable accessibility, high cost) which raises questions about its value for pretreatment assessment of liver fibrosis in patients with CHC[11,12]. Nowadays, many clinicians no longer cite LB as the gold standard but, at best, it can only be considered an imperfect standard for the staging of liver fibrosis[13]. It was this context that, in recent years, triggered a huge interest in the noninvasive assessment of liver fibrosis in patients with CHC. The introduction of a noninvasive methodology for the assessment of liver fibrosis as an alternative to LB in patients with CHC represents a major advancement in clinical hepatology[14]. Many of the noninvasive methods demonstrated accuracy to a considerable degree in identifying significant fibrosis, particularly cirrhosis, and consequently, noninvasive assessment of fibrosis is already a reality in patients with CHC[15]. Obviously, with the recent therapeutic development in CHC and reliable noninvasive diagnostic procedures available, LB has lost both its monopoly in the pretreatment assessment of fibrosis and the influence on decision making for antiviral therapy in patients with CHC.

CASE AGAINST LB

For the last 50 years, LB has been considered the gold standard for the staging of liver fibrosis in spite of its several shortcomings: intra- and interobserver variability in histopathological interpretation[16,17], sampling errors[18,19], and potentially life-threatening complications[20,21]. In clinical practice, we frequently encounter the intra- and interobserver variability in the staging of liver fibrosis[16,17]. Diagnostic errors made by nonspecialist pathologists were reported in > 25% of patients undergoing LB in academic centers[22,23]. According to a recent study[24], community pathologists understaged liver fibrosis in > 70% of cases with CHC. Several studies have shown that sampling errors occur when the LB specimen size is too small for an accurate estimation of fibrosis[18,19]. Both the length and the diameter of the biopsy core may affect the accuracy of fibrosis stage evaluation in patients with CHC[25,26]. Obviously, the shorter and thinner the samples are, the greater is the number of misclassifications of liver fibrosis. There is some controversy among pathologists in defining an adequate LB sample for an accurate staging of liver fibrosis. Some investigators[27] suggest that a sample of at least 15 mm in length and containing more than five portal tracts is adequate, while others recommend biopsy samples of 20 mm containing at least 11 portal tracts[26] or even larger samples, up to 25 mm[18]. Bigger is better[28], but at the price of an increased risk of severe complications[10,18]. However, it should be noted that, in clinical practice, few LB specimens reach an adequate length of 20 mm[29]. Furthermore, LB only samples an extremely small part of the whole organ (1/50 000) and therefore, there is a risk in the evaluation of lesions that are heterogeneously distributed throughout the entire liver[21]. LB may underestimate the amount of fibrosis, and cirrhosis could be missed in 10%-30% of cases[30]. Studies concerning fibrosis staging have also shown differences in one third of cases with CHC between LB samples obtained from the right and left lobes of the liver during laparoscopy[19]. Data on LB complications are heterogenous and contain wide variations in reported rate from one study to another[10,20,21,31-34]. Major complications include bleeding and bile peritonitis, with a reported mortality rate ranging from 0.03% to 0.1%[10,20,31,32,34]. It is worthwhile mentioning that both the transjugular route and ultrasound guidance approaches to LB do not significantly reduce the rate of major complications[35,36]. Complication rates are higher when LB is performed by less-experienced physicians[31,37]. In addition, LB is costly, variably available, poorly accepted by patients, and not suitable for repeated evaluation. The cost of an LB in the United States, United Kingdom and Australia varies between 1000 and 2000 USD, and it could go over 3000 USD if complications occur[12,38-40]. LB is not welcomed by patients and it may be refused by more than half of those with CHC[41]. LB is inappropriate for a dynamic evaluation of liver fibrosis over time, and recommendation to repeat biopsy every 3-5 years to follow up disease progression is certainly unrealistic, mainly due to patient nonadherence[40]. LB is contraindicated in the presence of coagulopathy and thrombocytopenia. Last but not least, the prevalence of CHC makes LB impossible in all patients with CHC who are candidates for antiviral therapy. It is these drawbacks of LB that have led to the development of noninvasive methods for the assessment of liver fibrosis in patients with CHC and, hopefully, to a major change in hepatology practice.

Nevertheless, LB has some well-recognized advantages for assessing fibrosis in CHC, such as direct measuring of liver fibrosis, well-established staging system, and evaluation of associated lesions (steatosis, iron deposition, inflammation, alcoholic liver disease, nonalcoholic fatty liver disease, metabolic syndrome), although these diagnostic advantages are counterbalanced by the aforementioned disadvantages.

CASE IN FAVOR OF NONINVASIVE METHODS

Noninvasive methods for detecting liver fibrosis may be divided in two main groups: serum markers of fibrosis and transient elastography (Fibroscan).

Serum markers for liver fibrosis are commonly divided into direct serum markers, which are directly linked to the modifications in extracellular matrix turnover produced by hepatic stellate cells during the process of fibrogenesis in the liver, and indirect serum markers which reflect alterations of the hepatic functions. The direct markers include glycoproteins (hyaluronate, laminin, YKL-40), collagen family (procollagen Ⅲ, type Ⅳ collagen), collagenases and their inhibitors (matrix metalloproteases, tissue inhibitory metalloprotease-1), and they are not routinely available in most clinical laboratories. The indirect markers are biochemical parameters determined in routine blood tests [platelet count, prothrombin time, aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio]. Serum markers for liver fibrosis may be used singly[42-45] or combining panels of direct or indirect serum markers and demographic parameters[46-55], with the aim of increasing the accuracy of single parameters. Some of them are patent-protected and commercially available: FibroTest® (Biopredictive, Paris, France) licensed under the name of Fibrosure® in the United States (LabCorp, Burlington, NC, United States)[51], Fibrometer® (BioLiveScale, Angers, France)[52], Hepascore (PathWest, University of Western Australia, Australia)[53], ELF® (Enhanced Liver Fibrosis Test, iQur Ltd, Southampton, United Kingdom)[54], and FibroSpect Ⅱ® (Promotheus Laboratory Inc. San Diego, Ca, United States)[55]. Among these, Fibrotest [α-2-macroglobulin, γ-glutamyl transpeptidase (GT), apolipoprotein A1, haptoglobin, total bilirubin, age, sex] is the most widely used and was validated by several studies on patients with CHC[56-63]. The reported accuracy of Fibrotest for significant fibrosis/cirrhosis expressed as area under receiving operating characteristic curve (AUROC) ranges from 0.74% to 0.87%[46,51]. To improve the performance of Fibrotest, its combination with Fibroscan has been suggested; with such a combination, one study reported AUROC of 0.88 for at least F2 (stage in the Metavir scoring system) and 0.95 for F3 or F4[56]. The sensitivity and specificity of serum-marker-based tests could also be improved by combining them using sequential algorithms. Thus, Sebastiani *et al*[64] combined AST/platelets ratio (APRI) with Fibrotest - a combination known as sequential algorithm for fibrosis evaluation biopsy - and found it to have an accuracy of 92.5% in the detection of fibrosis in CHC, obviating 81.5% of liver biopsies. APRI has a slightly lower performance than Fibrotest, with an accuracy between 60% and 82% for significant fibrosis and 60% and 88% for cirrhosis[46,64], but it is a simple cost-free readily available test in all hospital settings. Both Fibrometer (platelet count, hyaluronate, AST, α-2-macroglobulin, international normalized ratio, urea, age) and Hepascore (bilirubin, γGT, α-2-macroglobulin, hyaluronic acid, age, sex) showed good performance for detection of significant fibrosis[52,53,65].

There are several advantages of serum markers such as high applicability, with no risk for the patient and no contraindication; they can be performed and repeated in outpatient clinics; widespread availability; and inter-laboratory reproducibility[66]. However, there are some limitations of serum markers: none is liver specific; results are unreliable in comorbidities (hemolysis, Gilbert syndrome, rheumatoid arthritis); and they have poor performance in the diagnosis of intermediate stages of liver fibrosis[66]. Nevertheless, it is important to note that the performance of each noninvasive marker is evaluated against LB which is an imperfect gold standard, and the apparent failure of noninvasive markers to make an accurate distinction between different stages of intermediate fibrosis could be the consequence of misclassifications from biopsy[67,68].

Transient elastography (Fibroscan®, Echosens, Paris, France) measures liver stiffness in a volume at least 100 times greater than a standard LB sample, and therefore, may be more representative of the entire liver. Fibroscan is composed of an ultrasound transducer probe mounted on the axis of a vibrator; vibration is transmitted to induce an elastic shear wave that propagates through the liver. Pulse-echo ultrasound acquisition is used to measure the velocity of the shear wave, which is directly related to liver stiffness: the stiffer the liver, the faster the shear wave propagates. Results are expressed in kPa, and values range from 2.5 kPa to 75 kPa, with normal values < 5.5 kPa[69]. According to several studies, a cutoff value of 7.2-8.7 kPa defines significant fibrosis, and cirrhosis is diagnosed by a cutoff value of 12.5-14.5 kPa[70,71]. Fibroscan seems to be a reliable method for the diagnosis of significant fibrosis (AUROC 0.84) and cirrhosis (AUROC 0.95)[72,73]. Its combination with serum-based tests (Fibrotest, Fibrometer) increases the performance (but also the costs) for the diagnosis of significant fibrosis[56,71,72]. Among noninvasive methods for diagnosis of cirrhosis, Fibroscan has the highest level of performance[62,72,73], and its combination with serum markers does not increase accuracy[63,72].

Fibroscan has several advantages: it is painless; quick (< 5 min); highly reproducible, with results immediately available; inexpensive; and easy to perform in the outpatient clinic and at the bedside[66]. In addition, Fibroscan can be repeated for longitudinal disease monitoring, which is difficult, if not impossible, with LB. In cirrhotic patients, Fibroscan values correlate with portal pressure (based on the hepatic venous pressure gradient measurement), which is a reliable predictor of clinical outcomes[74-77], disease severity[78], and the risk of hepatocellular carcinoma[79]. Finally, Fibroscan and serum markers are well accepted by patients, therefore, they could be used as screening methods for the detection of liver fibrosis/cirrhosis in at-risk groups[80] and even in general population[81], while LB is unacceptable for screening purposes. Fibroscan measurement failure and unreliable results are due to limited operator experience[82], narrowed intercostal spaces[82], and obesity[82,83], although

this last problem seems to be overcome by a new specially designed probe[84-86]. Results are influenced by ALT flares[87,88], extrahepatic cholestasis[89,90], and congestive heart failure[91].

DISCUSSION

In the past, expert consensus guidelines on the management of CHC unanimously recommended routine LB before initiation of antiviral therapy[3-5,92,93]. Based on LB findings, treatment has often been advocated only for patients with at least moderate to severe stages of fibrosis (Metavir F2, F3 or F4), and withheld for those with no or minimal fibrosis (F0, F1)[6,93]. As a consequence, tens of thousands of patients were most likely denied proper antiviral therapy. More recent guidelines[8,94] recommend LB only in patients with CHC genotype 1 (SVR rate < 50%) in treatment decision making, and consider it unnecessary in those with genotype 2 or 3 who may have an SVR rate as high as 80%. The primary endpoint of antiviral therapy for CHC is achieving SVR - defined as undetectable serum HCV RNA at 24 wk after discontinuation of therapy. Viral eradication prevents disease progression, improves survival, and reduces health care costs associated with the management of complications. Thus, if viral clearance is the aim of antiviral therapy in CHC, then to what degree does an exact histopathological fibrosis stage established through biopsy still matter? With the new protease inhibitor (PI)-based triple therapy (addition of telaprevir or boceprevir to pegylated interferon and ribavirin) available and SVR rates approaching 75% in patients with CHC genotype 1[95,96], it is clear that LB has lost its importance in the recommendation of antiviral therapy.

During the past 10 years, an intensive debate has taken place between those in favor of LB and those who promote noninvasive methods for pretreatment assessment of liver fibrosis in patients with CHC. There is extensive literature showing the pros and cons of LB or noninvasive methods. As in chess, winning does not come easy for a supporter of noninvasive methods against a supporter of LB with a firmly rooted preference. Step by step, those in favor of non-invasive methods have gained ground, waiting for the final move: checkmate! Today, several noninvasive methods, ranging from serum marker assays to advanced imaging techniques, have proved to be excellent tools for the evaluation of liver fibrosis in patients with CHC. According to the latest European Association of the Study of the Liver clinical practice guidelines[97] and United Kingdom consensus guidelines[98] recommendations, noninvasive methods can be used instead of LB in patients with CHC to assess liver disease severity prior to antiviral therapy. It is therefore surprising that many experts in the field of hepatology and the most recent American Association for the Study of Liver Diseases 2011 practice guidelines[99] favor LB before therapy initiation, despite substantial improvement in treatment success rate for genotype 1 patients with PI-based triple therapy. The main reason against noninvasive methods for evaluation of liver fibrosis is their apparent failure to make an accurate distinction between different stages of intermediate fibrosis. It is important to note that the performance of each noninvasive method was evaluated in all studies by calculating the AUROC using LB as a reference standard. As LB is an imperfect standard, a perfect noninvasive method will never reach the maximum value (1.0)[100], and therefore, noninvasive methods are as inaccurate as LB for the assessment of fibrosis stage. Thus, the failure of noninvasive methods to discriminate between different stages of intermediate fibrosis could be the consequence of classification errors from histopathological findings of biopsy[67,68]. For clinicians, it is more important to know if their patients have no/mild or advanced fibrosis/cirrhosis, rather than the exact pathological scoring system through LB, and this could be easily achieved by means of noninvasive methods. Taking into account that all recent international guidelines[97-99] recommend treatment with PI-based triple therapy in all patients with CHC genotype 1, provided that they have no contraindications to peg-interferon and ribavirin, the need to stage liver fibrosis accurately is decreasing in treatment decisions.

The final move - checkmate to LB - is, therefore, possible once the rate of SVR has reached 75% with PI-based triple therapy for patients with CHC genotype 1. Consequently, it is clear that in the era of PI-based triple therapy and other new potent direct-acting agents in the pipeline, the information obtainable from LB has little, if any, influence on treatment decisions. It should be underlined that in this article, checkmate to LB in patients with CHC refers strictly to cases with no need for this invasive and risky procedure in therapeutic decision making. With PI-based triple therapy already available in many countries, and an allocation system probably based mainly on medical need (therapy for those likely to develop complications in the next few years), noninvasive methods with the highest accuracy for detecting severe fibrosis/cirrhosis used as an alternative to LB for pretreatment assessment of liver fibrosis in patients with CHC are now part of routine clinical practice. Fibroscan or any patented biomarkers (Fibrotest, Fibrometer and Hepascore) have recently been recommended for first-line staging of liver fibrosis[101] before deciding on antiviral therapy. However, the adoption rates of noninvasive methods by hepatologists differ from country to country. In France, a survey of 546 hepatologists revealed that 81% of them used noninvasive methods[102], while in the United States, despite the aforementioned shortcomings of LB, there is still significant resistance to accepting noninvasive methods as an alternative to biopsy. We believe that sooner or later this will change, and the requirement of LB prior to starting antiviral therapy in patients with CHC will be reassessed.

In conclusion, in the era of PI-based triple therapy and other new potent direct-acting agents on the horizon that can achieve SVR rates approaching 100%, the time has come to move forward to risk-free noninvasive

methods for the patient, leaving LB behind in the evaluation of liver fibrosis in decision making for CHC antiviral therapy. In other words, checkmate to LB?

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REVIEW

Treatment of locally advanced rectal cancer: Controversies and questions

Atthaphorn Trakarnsanga, Suthinee Ithimakin, Martin R Weiser

Atthaphorn Trakarnsanga, Martin R Weiser, Colorectal Service, Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, NY 10065, United States
Atthaphorn Trakarnsanga, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
Suthinee Ithimakin, Division of Medical Oncology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
Author contributions: Trakarnsanga A and Ithimakin S wrote the manuscript; Weiser MR critically revised the manuscript and oversaw intellectual content.
Correspondence to: Martin R Weiser, MD, Colorectal Service, Department of Surgery, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, Room C-1075, New York, NY 10065, United States. weiser1@mskcc.org
Telephone: +1-212-6396698 Fax: +1-212-7943198
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Abstract

Rectal cancers extending through the rectal wall, or involving locoregional lymph nodes (T3/4 or N1/2), have been more difficult to cure. The confines of the bony pelvis and the necessity of preserving the autonomic nerves makes surgical extirpation challenging, which accounts for the high rates of local and distant relapse in this setting. Combined multimodality treatment for rectal cancer stage Ⅱ and Ⅲ was recommended from National Institute of Health consensus. Neoadjuvant chemoradiation using fluoropyrimidine-based regimen prior to surgical resection has emerged as the standard of care in the United States. Optimal time of surgery after neoadjuvant treatment remained unclear and prospective randomized controlled trial is ongoing. Traditionally, 6-8 wk waiting period was commonly used. The accuracy of studies attempting to determine tumor complete response remains problematic. Currently, surgery remains the standard of care for rectal cancer patients following neoadjuvant chemoradiation, whereas observational management is still investigational. In this article, we outline trends and controversies associated with optimal pre-treatment staging, neoadjuvant therapies, surgery, and adjuvant therapy.

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Key words: Rectal cancer; Neoadjuvant chemoradiation; Response; Treatment; Staging; Recurrence

Peer reviewers: Abdul-Wahed Meshikhes, MD, FRCS, Chairman and Consultant Surgeon, Department of Surgery, King Fahad Specialist Hospital, Amir Bin Thabit St, Dammam 31444, Eastern Province, Saudi Arabia; Marty Zdichavsky, MD, Department of General, Visceral and Transplant Surgery, University Hospital Tübingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany; Herwig R Cerwenka, Professor, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria

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INTRODUCTION

The mainstay of treatment for colorectal cancer is surgery. Complete, margin-negative resection confers the greatest chance for cure. However, chemotherapy and radiation have important roles in assuring long-term, recurrence-free survival. Colon cancer and extraperitoneal rectal cancers (lesions above the anterior peritoneal reflection, or 12-15 cm from the anal verge) are generally treated with surgery followed by adjuvant chemotherapy; these patients are believed to be at high risk for harboring micrometastasis and, therefore, of developing of recurrent disease. Mid to low rectal cancers lying below

the anterior peritoneal reflection and extending through the rectal wall, or involving locoregional lymph nodes (T3/4 or N1/2), have historically been more difficult to cure. The confines of the bony pelvis and the necessity of preserving the autonomic nerves makes surgical extirpation challenging, which accounts for the high rates of local and distant relapse in this setting. The Gastrointestinal tumor study group and National surgical adjuvant breast and bowel project trials demonstrated that chemoradiotherapy following surgical resection could reduce local recurrence from 55% to 33%, with significantly prolonged disease-free survival (DFS)[1,2]. This was the basis for the National Institute of Health consensus statement in 1990 recommending combined modality therapy for Stage Ⅱ and Ⅲ rectal cancer[3]. In the 20 years since, there have been considerable advances in treatment.

We will outline current trends and controversies in the treatment of locally advanced rectal cancer. The topics to be covered include staging, neoadjuvant therapies, surgery, and adjuvant therapy.

OPTIMAL CLINICAL STAGING

Pre-treatment evaluation, including accurate staging, is critical when planning treatment for rectal cancer patients. The mainstay of treatment is surgery, which runs the gamut from local excision to radical resection with or without chemoradiation. Accurate staging is the first crucial, necessary step in optimizing therapy, maintaining quality of life, and limiting over- and under-treatment.

Most rectal cancers are diagnosed at colonoscopy, usually after patients present with asymptomatic anemia or change in bowel habits. Histologic confirmation is important, as benign stricture and inflammatory conditions may mimic malignancy. Full colonoscopy is necessary in order to identify synchronous polyps and malignancies, which occur in about 30% and 3%-5% of cases, respectively[4,5]. If the colon cannot be fully evaluated preoperatively due to colonic obstruction, it is recommended at 3-6 mo postresection[6].

Once a patient is diagnosed with rectal cancer, the most commonly ordered imaging study is computed tomography (CT). When properly performed with oral and intravenous contrast, CT provides important information regarding extent of disease. Although it is not a very accurate imaging modality for determining the degree of primary tumor penetration into the rectal wall or involvement of locoregional lymph nodes, CT scanning of the chest, abdomen and pelvis accurately identifies distant metastatic disease in the lung, liver, pelvic and periaortic lymph nodes.

Staging of the primary rectal lesion begins with a detailed digital rectal examination (DRE). This confers important information about the primary lesion, including its location with respect to the top of the anorectal ring (external sphincter complex) as well as its mobility. The degree of tumor fixity in the pelvis corresponds to depth of penetration through the rectal wall. Mobile lesions are often limited to the mucosa, submucosa or muscularis propria, whereas tethered lesions are likely to extend into the perirectal fat (mesorectum). A fixed lesion may signify extension of tumor into surrounding anatomic structures such as the seminal vesicles, prostate or vagina (T4 lesions). Rigid proctosigmoidoscopy provides the best estimate of tumor distance from the anal verge.

Endorectal ultrasonography (ERUS) and magnetic resonance imaging (MRI) are the most accurate tools for assessing T and N stage. ERUS is advantageous in imaging small, superficial tumors, whereas MRI is useful in evaluating bulky tumors that extend to the circumferential margin (CRM)[7]. Based on the results of large meta-analyses, the accuracy of ERUS in determining T and N stage is 87% and 74%, respectively[8]. The accuracy of MRI in determining T and N stage is 71%-91% and 45%-79%, respectively[9], similar to that of ERUS. Introduction of endoluminal coil MRI improves imaging resolution of the rectal wall. However, the true strength of MRI lies in phased-array MRI, which facilitates accurate assessment of tumor encroachment of the CRM, or invasion into surrounding anatomic structures[10]. Both MRI and ERUS are less accurate in assessing locoregional lymph node metastasis. Morphology, such as irregular border or heterogeneous intensity, is often more predictive than lymph node size[11]. It is important to remember that 18% of nodal metastases occur in lymph nodes measuring less than 5 mm[12]. Rather than being competing modalities, however, ERUS and MRI are often complimentary in the process of rectal cancer staging. ERUS is particularly useful in the staging of early rectal cancers; it also has the advantage of low cost, and can be performed quickly in the outpatient office. Phased-array MRI is much more expensive and not as readily available; however, it affords a comparatively greater field of vision, enhancing the ability to identify at-risk CRM and invasion of adjacent organs.

CHEMORADIATION: POSTOPERATIVE *VS* PREOPERATIVE

A paradigm shift in preoperative radiotherapy (RT) was introduced by the Swedish Rectal Cancer Trial in 1997. This study randomized 1168 patients to receive either one week of RT followed by surgery, or surgery alone. Compared with surgery alone, patients who received preoperative RT had reduced local recurrence (11% *vs* 27%, $P < 0.001$) and prolonged survival [5-year overall survival (OS) of 58% *vs* 48%, $P = 0.004$][13]. At a median follow-up of 13 years, the benefits in terms of local recurrence (9% *vs* 26%, $P < 0.001$) and OS (38% *vs* 30%, $P = 0.008$) remained significant in patients who received preoperative RT[14]; however, these patients did experience more gastrointestinal complications and had a higher rate of hospitalization over the 6-mo period following surgery[15].

Total mesorectal excision (TME) involves sharp dis-

section along the embryonic planes between the visceral and parietal layers of the endopelvic fascia. This ensures complete removal of locoregional lymph nodes contained within the mesorectum, while preserving the autonomic nerves and limiting blood loss. Multiple retrospective and cohort studies have shown that TME is associated with lower rates of local recurrence compared to the less optimal blunt surgical technique. The Dutch TME trial (published in 2003) was the first to compare the results of TME with and without preoperative short-course RT. Of 1861 accrued patients, 924 and 937 were randomized to receive either preoperative radiation followed by TME, or TME alone. Local recurrence was significantly lower in patients, who received preoperative RT plus TME (2.4% *vs* 8.2%, $P < 0.001$), but there was no difference in OS[16]. Long-term follow-up showed lower recurrence rates in the preoperative RT arm, especially in the subgroups of patients with nodal involvement, patients with tumor located between 5-10 cm from the anal verge, and patients with free CRMs[17].

While preoperative short-course radiotherapy consisting of 25 Gy in 5 fractions has been the favored treatment in Northern Europe and Scandinavia, in North America and in some European countries long-course chemotherapy has been the treatment of choice, based in part on the results of the Gastrointestinal Tumor Study Group and National Surgical Adjuvant Breast and Bowel Project (NSABP) trials. With theoretical advantages of better tolerance and increased efficacy, many centers moved toward preoperative neoadjuvant chemoradiation: 50.4 Gy delivered in 28 fractions, and concurrent fluoropyrimidine-based chemotherapy. The majority of patients receiving chemoradiotherapy obtain tumor downstaging (in which the final pathologic stage at time of surgery is lower than the initial clinical stage at time of presentation)[18]. Indeed, as many as 15%-20% of patients will have a complete pathologic response to treatment, with no viable tumor cells noted in the resected rectum. Tumor downsizing may facilitate complete tumor resection and, in the setting of low-lying tumors, may alter the surgical plan by making a sphincter-saving procedure possible[19,20].

Although two prospective, randomized controlled trials comparing preoperative and postoperative chemoradiation failed to accrue; NSABP R03[21] and the Intergroup 1047 trial[22] in the United States, the German Rectal Cancer Study Group successfully completed such a trial. The German CAO/ARO/AIO 94 trial[19] compared preoperative and postoperative long-course chemoradiation for T3 or T4 and/or node-positive rectal cancer. Chemoradiotherapy consisted of 50.4 Gy in 28 fractions with concurrent infusional fluorouracil (1000 mg/m^2 per day for 5 d in the first and fifth week of radiation). Four hundred twenty-one and 402 patients were randomly allocated to receive preoperative and postoperative chemoradiotherapy, respectively. There was 6% local recurrence in the preoperative group and 13% in the postoperative group ($P = 0.006$). Grade 3 or higher acute and long-term toxicity occurred significantly less frequently in patients who received neoadjuvant chemoradiation ($P = 0.001$ and $P = 0.01$, respectively). However, the rates of sphincter preservation, DFS and OS did not differ between the two groups.

Comparison of long- and short-course radiotherapy was the aim of a Polish randomized study[23,24] of patients with T3/4 mid to low rectal cancer. The results demonstrated higher rates of complete pathologic response in the group of patients receiving long-course chemoradiotherapy: 16% and 1% had complete pathologic response in the long-course and short-course chemoradiotherapy groups, respectively. Although rates of sphincter preservation were similar in both study groups, patients receiving long-course chemoradiotherapy had a 4% rate of positive CRM at time of surgery, compared with 13% in the short-course group ($P = 0.017$). However, there was no significant difference in local recurrence, DFS or OS.

The recently reported MRC CR07 and NCIC-CTG C016 multi-center randomized study[25] of 1350 patients compared the outcomes of preoperative short-course RT *vs* initial surgery followed by selective postoperative chemoradiation in patients with positive CRM. The primary outcome was local recurrence. This study demonstrated a significant decrease in local recurrence in patients receiving preoperative short-course RT (hazard ratio 0.39, $P < 0.0001$), which was associated with a 6% absolute improvement in DFS at 3 years ($P = 0.03$). Again, these data demonstrate the superiority of preoperative chemoradiotherapy.

The above data indicate that both preoperative long-course and short-course radiotherapy followed by proper TME provide excellent local control. The advantages of long-course chemoradiotherapy include tumor downsizing and downstaging, which may alter the surgical treatment plan in favor of a sphincter-preserving procedure. Long-course chemoradiation is associated with higher acute toxicity than short-course (18.2% *vs* 3.2%, respectively)[23]. On the other hand, short-course RT may lead to more long-term complications secondary to higher dose per fraction. In the United States, long-course chemoradiotherapy consisting of 5040 cGy, delivered concurrently with 5-fluorouracil (5FU) chemotherapy, is the most common regimen[6].

With improvements in surgical technique, including TME, the utility of chemoradiation in early T3 lesions without at-risk CRM has been called into question. A study of 95 T3N0 rectal cancer patients undergoing TME demonstrated less than 10% local recurrence rate without adjuvant therapy[26]. A pooled analysis of 3791 patients from several randomized studies showed no difference, in terms of OS, in T3N0 rectal cancer patients who received surgery plus chemotherapy with, or without, radiation (85% *vs* 74%-80%, respectively). Moreover, local relapse was similar among stage Ⅱ patients undergoing surgery alone (14%) or surgery plus radiotherapy (12%)[27]. It has been suggested that patients at lower

risk of local recurrence, e.g., those with proximal T3N0 lesions with clear margin, may be adequately treated by surgery and only adjuvant chemotherapy. However, correctly assessing T and N stage remains problematic. Two studies have reported the limitations of ERUS or MRI in determining accurate nodal stage, with false negative rates of up to 22%-28%[28,29]. Therefore, preoperative chemoradiation for clinical T3N0 rectal cancer patients should be considered by weighing the risk of unnecessary treatment against the possibility that the patient may ultimately require postoperative chemoradiation, which is associated with lower local control and higher toxicity than preoperative chemoradiation. Currently, preoperative chemotherapy remains the standard of treatment for T3N0 patients based on the principle that overtreatment is less harmful than undertreatment.

MAXIMIZING RESPONSE TO NEOADJUVANT CHEMORADIATION

Fluoropyrimidine-based chemotherapy synergizes with long-course radiotherapy. A 2 × 2 study of the European Organization for Research and Treatment of Cancer (EORTC) 22921 trial[30] assessed the efficacy of adding chemotherapy to RT, with or without adjuvant (postoperative) chemotherapy. The addition of chemotherapy at some point in the treatment regimen, either preoperatively or postoperatively, conferred a significant benefit in terms of local control. RT is fundamental to neoadjuvant treatment of rectal cancer, resulting in increased pathological response rate and better local control. However, it has no effect on distant metastasis. These findings imply that fluorouracil acts as a potent radiation sensitizer but has no significant eradicating impact on micrometastatic disease. In an attempt to improve response rates and reduce distant metastasis, new preoperative strategies are being investigated; these include combinations of new chemotherapeutic agents used concurrently with RT as well as induction chemotherapy. Currently efforts are being made to integrate novel agents in combination with RT, using the pathologic complete response (pCR) rate as a surrogate endpoint. Pathological CR has demonstrated correlation with clinical outcomes, i.e., relapse-free survival, DFS and OS[31,32].

Capecitabine, an oral pro-drug of fluoropyrimidine designed to enable selective 5FU activation in tumor tissue, has been studied as an agent used concurrently with RT. Several phase Ⅰ / Ⅱ studies have revealed a pCR rate of 7%-31% with acceptable toxicities[33-36]. Two randomized controlled trials showed capecitabine to be non-inferior to 5FU in the perioperative treatment of stage Ⅱ-Ⅲ rectal cancer. The NSABP R-04[37] compared the efficacy of continuous infusion 5FU to capecitabine delivered concurrently with preoperative RT, with or without oxaliplatin, in 1608 patients. The rates of pCR were 19% and 22% in patients who received 5FU and capecitabine, respectively (P = 0.12). Long-term outcomes of DFS and OS are awaited. Another phase Ⅲ trial from Germany[38] demonstrated that capecitabine, used in the neoadjuvant or adjuvant setting, is not inferior to 5FU in terms of 5-year OS (capecitabine 76%; 5FU 67%, P = 0.053). The use of oral capecitabine (825 mg/m^2 taken twice daily) throughout the course of radiation has been an alternative option; it is not inferior to infusional fluoropyrimidine in terms of efficacy or toxicity, and has the potential advantage of convenience for the patient.

The results of two phase Ⅲ studies studying combined oxaliplatin and fluoropyrimidine used concurrently with radiotherapy have also been reported. The ACCORD12 trial compared CRT using capecitabine + 45 Gy RT to capecitabine + oxaliplatin (CAPEOX) + 50 Gy RT[39]. This study showed pCR rates of 13.8% and 18.8% in the capecitabine/RT and CAPEOX/RT groups, respectively (P = 0.11). Another phase Ⅲ study from Italy investigated the efficacy of adding weekly oxaliplatin to protracted infusion of 5FU, concurrently with radiation, in locally advanced rectal cancer patients[40]. There was no difference in the pCR rate (16% in the 5FU group *vs* 15% in the 5FU + oxaliplatin group, P = 0.98). Interestingly, fewer patients in the 5FU + oxaliplatin arm were found to have metastatic disease after neoadjuvant chemoradiation (0.5% *vs* 3%, P = 0.014); this data may imply that oxaliplatin is potentially efficacious in controlling micrometastasis. Another trial of NSABP R-04[37], reported at the 2011 American Society of Clinical Oncology Annual Meeting, demonstrated that oxaliplatin does not improve pCR rates (19.1% *vs* 20.9%, P = 0.46), but significantly increases toxicity. However, neither randomized controlled trial showed any improvement in acute endpoints, i.e., pCR. At this time, single agent fluoropyrimidine, either infusional 5FU or capecitabine used concurrently with pelvic radiation, remains the standard of care in stage Ⅱ and Ⅲ rectal cancer.

Attempts have been made to integrate molecularly targeted therapy with preoperative combined modalities, in order to improve the efficacy of standard neoadjuvant chemoradiation. The addition of cetuximab to capecitabine was evaluated in a phase Ⅰ / Ⅱ trial in which only 5% of patients reached pCR; however, there was no unexpected toxicity[41]. Another study from Germany also reported a suboptimal pCR rate (9%) after preoperative cetuximab + capecitabine, oxaliplatin and radiotherapy[42]. Data on preoperative bevacizumab are limited to a few phase Ⅰ studies. However, in contemplating treatment with bevacizumab, the known adverse effects of this monoclonal antibody (such as bowel perforation, bleeding, and impaired surgical wound healing) must be considered.

Induction chemotherapy followed by neoadjuvant chemoradiation was considered a potential approach to controlling micrometastasis. A phase Ⅱ United Kingdom trial, using this new strategy of neoadjuvant chemotherapy with CAPEOX prior to CRT and surgery, has been reported[43]. One hundred five individuals, identified by high-resolution MRI as poor-risk rectal cancer patients, were enrolled. The definition of "poor-risk"

entailed one of the following features: CRM threatened or involved by tumor, low-lying tumor at or below the levators, T3 tumor with radial margin > 5 mm, T4 lesion, or N2 disease. The results showed the feasibility of this approach, with overall rates of 5-year and DFS 75% and 68%, respectively. In the setting of locally advanced rectal tumors, these are very impressive statistics.

A Spanish trial[44] was conducted to compare this new strategy with the more conventional approach of neoadjuvant chemoradiation followed by surgery and adjuvant chemotherapy. One hundred eight patients were randomized to receive 4 cycles of CAPEOX for the induction period, followed by neoadjuvant chemoradiation and then surgery; or else to receive neoadjuvant chemoradiation followed by surgery, and subsequently 4 cycles of adjuvant CAPEOX. There was no difference in pCR rate (13% *vs* 14%). Significantly less toxicity and a greater ability to tolerate chemotherapy throughout the schedule were found in patients who received induction chemotherapy. Another phase Ⅱ study from Memorial Sloan-Kettering Cancer Center[45] was conducted to determine the efficacy of induction chemotherapy, followed by chemoradiotherapy and then surgery, for stage Ⅲ or potentially resectable stage Ⅳ rectal cancer patients. Thirty-four patients were enrolled. Seventeen of 27 patients who ultimately underwent TME had > 90% pathological tumor response. At a median follow-up of 25 mo, no local recurrence was identified. Three-year DFS and OS were 63% and 100%, respectively, in patients with resected stage Ⅲ rectal cancer. This study showed a promising clinical outcome with tolerable toxicity. We believe that this approach will become one of the mainstays of treatment for patients with locally advanced rectal cancer in the future, but the data must be borne out in larger phase Ⅲ studies.

PREDICTORS OF RESPONSE TO NEOADJUVANT THERAPY

Several parameters associated with tumor response to neoadjuvant chemoradiation have been identified. Some studies have concluded that lower pre-treatment carcinoembryonic antigen (CEA) level is associated with better response to treatment[46-50]. The cut-off CEA levels associated with response rate were 2.5[46], 3[47], and 5 ng/mL[48-50]. However, because the pre-treatment CEA level in a majority of patients is normal and the cut-off level inconclusive, the applicability of CEA in predicting treatment response remains unclear.

Location of tumor, including tumor distance from the anal verge and the extent of luminal involvement by tumor, has also been examined. Bulky T3 lesions are less likely to show significant downstaging[51]. In a study of 247 patients, T3 tumors with > 2.5 mm extension into the mesorectum demonstrated a lesser response to therapy, with a low degree of downstaging[51]. It cannot be determined if this is merely attributable to tumor volume or to actual tumor biology. Predictably, this study also noted that poorly differentiated histology and metastatic disease were associated with limited response to treatment. Similarly, other reports have found that there is comparatively less downsizing in lesions with > 60% circumferential involvement. Interestingly, in this study lesions located higher than 5 cm from the anal verge were more likely to show a significant response to neoadjuvant chemoradiation[46].

In the past decade, many studies have attempted to identify correlations between immunohistochemical biomarkers such as Ki-67, p53, p21, bax/bcl2, epidermal growth factor receptor, thymidylate synthase, and tumor response after neoadjuvant chemoradiation. Currently, however, these studies remain inconclusive[52-54].

RESTAGING AFTER NEOADJUVANT CHEMORADIATION

Identifying the 15%-20% of patients who achieve a complete response to neoadjuvant therapy remains a challenge. Because of their fundamental roles in the neoadjuvant chemoradiation setting, DRE, endoscopy, ERUS, CT, MRI and positron emission tomography (PET) have been proposed as investigational tools to determine tumor response to treatment. The problem is that none of these modalities are capable of accurately predicting pCR[55], the reason being that fibrosis and inflammation caused by radiation have a deleterious affect on accuracy. A study from our institution[56] demonstrated that DRE is an inaccurate method for determining response to treatment after neoadjuvant chemoradiation; the overall concordance between DRE and pathologic response following neoadjuvant chemoradiotherapy was only 22%. A study from Italy showed that only half of patients who were defined as having complete response on endoscopic biopsy had true pCR according to pathology from surgical resection[55].

The accuracy of ERUS and MRI in the setting of post-neoadjuvant chemoradiation is also limited. A prospective study compared the accuracy of CT, MRI and ERUS *vs* pathologic assessment in determining clinical T and N stage in 90 patients receiving long-course neoadjuvant chemoradiation. The accuracy of these imaging modalities in determining T stage was low (37% by CT, 34% by MRI, and 27% by ERUS); most of this was due to over-staging. The rate of accuracy in nodal staging was 62% by CT, 68% by MRI and 65% by ERUS[57].

Because of the limited accuracy of all existing imaging modalities in staging rectal cancer post-neoadjuvant chemoradiation, several groups have investigated novel imaging methods. Diffusion-weighted MRI is a functional MRI imaging technique with better accuracy than that of conventional MRI[58-60]. However, the role of this emerging method is still investigational, and more studies with larger numbers of patients are awaited. In the meantime, repeat CT of the chest, abdomen and pelvis is useful in identifying development of interval metastatic disease, the presence of which could alter the surgical plan.

PET is a functional imaging study. Theoretically, the change of activity in the tumor should relate to the treatment response. Support to this idea, there are some studies demonstrated the relative change of maximum standardized uptake value relate to tumor response[61-63]. On the contrary, the data from another study demonstrated 63% sensitivity and 74.4% specificity of PET to predict patients with complete response[64]. While, PET is sometimes utilized in the process of restaging, the current data on the efficacy of this modality is inconsistent and limited; thus we do not routinely use PET as a restaging tool.

OPTIMAL TIMING OF SURGERY AFTER NEOADJUVANT THERAPY

In an attempt to increase response to chemoradiation, some groups have investigated lengthening the interval between chemoradiotherapy and surgery. Traditionally, surgery has been recommended 6-8 wk after neoadjuvant chemoradiation[65-69] to allow for tumor regression without extensive fibrosis. Indeed, several studies have shown that extending the period between chemoradiation and surgery may increase rates of complete pathologic response without increasing perioperative complications[63-65]. However, the effect of a longer interval on long-term outcome has yet to be defined. A well-designed multicenter prospective trial of increasing the interval between neoadjuvant chemoradiation and surgery is ongoing[70]. It is unclear if this will impact outcome; nevertheless, it may be of significance in the development of nonoperative strategies (see below). Some have also proposed adding chemotherapy to the waiting period, in an attempt to treat potential micrometastatic disease[71,72]. Habr-Gama *et al*[71] have reported the preliminary results of a study adding chemotherapy in the interval between neoadjuvant chemoradiation and surgery, which demonstrate an increased rate of complete clinical response.

MINIMALLY INVASIVE SURGERY FOR LOCALLY ADVANCED RECTAL CANCER

Laparoscopic surgery for rectal cancer has been proven feasible, but it is not yet a standard treatment in the United States. The United Kingdom Medical Research Council Trial of Conventional *vs* Laparoscopic-Assisted Surgery in Colorectal Cancer trial[73] demonstrated equivalent long-term recurrence and survival results in rectal cancer patients who did not receive neoadjuvant treatment. The Comparison of Open *vs* Laparoscopic surgery for Mid and Low Rectal Cancer after Neoadjuvant Chemoradiotherapy trial[74] is a prospective randomized trial that includes 340 patients with locally advanced rectal cancer receiving neoadjuvant chemoradiotherapy, randomized to laparoscopic and open groups in a ratio of 1:1. The conversion rate is only 1.2% (2 in 170 patients). From the short-term results, operative time is significantly longer in the laparoscopic group, but estimated blood loss was significantly less. The rate of positive circumferential resection margin did not differ significantly between the two groups; nor did distal resection margin. The long-term oncological outcomes are awaited.

PREDICTORS OF RECURRENCE AND SURVIVAL

Several factors are reportedly predictive of recurrence and survival. Some of these features are the same in patients who receive or do not receive neoadjuvant chemoradiation. Some differ.

Post neoadjuvant chemoradiation CEA level

It has been proposed that post-neoadjuvant chemoradiation CEA levels < 2.5 ng/mL[75] and 5 ng/mL[76] are associated with significantly better DFS and OS. Another study, however, discerned no correlation[77]. Moreover, most studies focusing on correlations between predictors and long-term outcomes do not focus on CEA levels[77-79]. Thus, application of this parameter in attempting to predict recurrence and survival remains inconclusive.

Tumor response and nodal status after neoadjuvant chemoradiation

The pathologic response of tumor to treatment is one of the most significant prognostic factors in rectal cancer[18,80]. In terms of recurrence and survival, pCR is most strongly correlated with excellent outcome[80-85]. Several tumor regression grading systems classify tumor response following neoadjuvant chemoradiation in patients with pathologic partial response. All of these grading systems are consistent and demonstrate a strong association between pathologic response and outcome[18,32,79,83]. In the setting of neoadjuvant chemoradiation, preoperative treatment reduces lymph node yield at the time of surgery. However, the ratio of positive lymph nodes to total lymph nodes is prognostic, and may in fact be a more useful prognostic factor than total number of lymph nodes culled following neoadjuvant chemoradiation[83]. Several studies have associated positive pathological lymph node (ypN) status with poor prognosis[30,77-83].

Distal resection margin

Historically, the standard guidelines have recommended a distal resection margin (DRM) of 4-5 cm from the distal edge of tumor[6,86]. However, several reports have found that a DRM < 2 cm does not increase recurrence rates[87,88] or negatively impact survival[88]. In the setting of low rectal cancer located < 5 cm from the anal verge, 1-2 cm may be acceptable[6]. Especially following preoperative treatment with neoadjuvant chemoradiation, the 1-2 cm DRM rule seems to be less important than obtaining a clear resection margin[89,90].

CRM

The standard cut-off point of the CRM is still a matter of controversy. Most published studies have used < or = 1 mm[91-93], 2 mm[94-96], or 5 mm[7,97] as acceptable cut-off points in chemoradiotherapy-naïve patients. All reported significantly higher recurrence rates and shorter survival in patients with CRM < or = 1 mm[91-93], 2 mm[94-96], or 5 mm, respectively[7,98]. Therefore, in patients who have not received neoadjuvant chemoradiation and whose pathology indicates a close CRM (less than 1-5 mm), adjuvant chemoradiation should be strongly considered regardless of other pathological results[90,98]. In patients who have already received neoadjuvant chemoradiation, adjuvant chemotherapy should be considered[95-97].

Perineural invasion and lymphovascular invasion

These are poor prognostic factors in colon and rectal cancer in general. Even with neoadjuvant chemoradiotherapy, Perineural invasion and lymphovascular invasion remain predictors of poor outcome[20,97].

Acellular mucin pools

Acellular mucin pools (AMP) has been found in 11%-27% of surgical cases after neoadjuvant chemoradiation[99,100]. The question is whether AMP after neoadjuvant chemoradiation should be considered residual disease or not. Several reports have concluded that AMP does not have a significant impact on outcome[99-101]. The College of American Pathologists has recommended that AMP not be interpreted as residual disease[102].

POSTOPERATIVE ADJUVANT CHEMOTHERAPY: IS IT NECESSARY?

There is a high risk of local recurrence and distant metastasis in stage Ⅱ and Ⅲ rectal cancer patients treated by surgery alone. The role of adjuvant chemotherapy is to eradicate micrometastatic disease. Patients with T3 or node-positive disease who are initially treated by transabdominal resection should receive six months of adjuvant therapy, consisting of a "sandwich regimen" of two-month fluoropyrimidine-based chemotherapy followed by concurrent chemotherapy and radiation, followed by another 2 mo of chemotherapy.

Previously, the standard adjuvant chemotherapy regimen for rectal cancer was 5FU with or without leucovorin. The use of combination oxaliplatin, 5FU and leucovorin (FOLFOX4) or capecitabine in adjuvant treatment has been extrapolated from data available for colon cancer. According to the MOSAIC trial[103], FOLFOX4 compared to 5FU + leucovorin improved DFS and OS in stage Ⅲ colon cancer patients. The X-ACT study[104] showed that, in terms of DFS and OS, the efficacy of capecitabine in adjuvant treatment of stage Ⅲ colon cancer was comparable to that of 5FU + leucovorin.

There has been much consideration of the need for adjuvant chemotherapy in patients who have already received preoperative chemoradiation. The EORTC 22921 study[28,105] assessed the value of adjuvant chemotherapy in patients undergoing preoperative CRT followed by surgery. Patients were allocated into four arms: preoperative RT; preoperative CRT; preoperative RT + adjuvant chemotherapy; and preoperative CRT + adjuvant chemotherapy. No difference in OS was found between the groups receiving preoperative and postoperative chemotherapy (P = 0.12). The addition of chemotherapy after preoperative CRT did not impact rates of local recurrence or survival. Of concern, however, is the fact that only 42.9% of assigned patients adhered to their postoperative chemotherapy regimens. DFS and OS benefits were shown in the subgroup of patients with pathological T (ypT) 0-2 cancers[106]. These results indicate that patients who do obtain downstaging after CRT may be more likely to derive a survival benefit from adjuvant chemotherapy as well.

Given that most of the patients in EORTC 22921 did not receive adequate doses of adjuvant chemotherapy, we cannot assume from this study that adjuvant chemotherapy is definitely not beneficial to patients receiving neoadjuvant chemoradiation. To date, preoperative chemoradiation followed by surgery and adjuvant chemotherapy remains the standard of practice in the United States for treating stage Ⅱ and Ⅲ rectal cancers[6] regardless of final pathological results. Options for adjuvant chemotherapy include four months of FOLFOX, capecitabine, or 5FU + leucovorin.

CLINICAL COMPLETE RESPONSE: CAN SURGERY BE AVOIDED?

Habr-Gama *et al*[107] have proposed a definition of clinical complete response (cCR), based on the findings of clinical and endoscopic examination, as follows: (1) whitening of the mucosa in an area of the rectal wall; (2) any associated telangiectasia; (3) scarring of the rectal wall (which manifests as a slight stiffness of the wall during insufflation); and (4) if tumor cannot be felt or seen. These parameters are subjective, however, and require more clinical substantiation. Moreover, cCR may not be equivalent to pCR. Surgery is still the standard of management following neoadjuvant chemoradiation[6,86].

There have been studies of local excision as an option for patients with cCR after neoadjuvant chemoradiation[108,109]. However, complete response of the primary tumor cannot predict response in regional lymph nodes, which are involved in 7%-17% of patients who have cCR of the primary tumor[110-112]. For this reason local excision may not be adequate treatment of these patients. In the setting of low-lying rectal cancer, sphincter-preserving procedures (intersphincteric dissection with coloanal anastomosis) may be performed in patients who obtain a tremendous response from neoadjuvant chemoradiation[20].

Habr-Gama *et al*[113] have also reported very interesting long-term outcomes of observational management in patients with cCR following neoadjuvant chemoradiation. They selected the 122 of 361 patients who received

neoadjuvant chemoradiation and were considered to have cCR. These patients had very low rates of both local recurrence and distant metastasis (5% and 7%, respectively). Isolated local recurrences confined to the rectum developed in 5% of patients over a median follow-up period of 59.9 mo[114]. Significantly more data from larger studies will be required to evaluate this approach. Nevertheless, observational management may be an alternative choice for some carefully selected patients.

CONCLUSION

Concurrent chemoradiotherapy is widely accepted as an effective way to achieve local control and survival in patients with locally advanced rectal cancer. The accuracy of preoperative staging is crucial in preventing under- or over-treatment. ERUS and MRI are suitable imaging tools for local preoperative staging, while CT and PET are helpful in evaluation of metastasis. Fluoropyrimidine-based chemotherapy (either 5FU or capecitabine) used concurrently with radiotherapy is the standard treatment. The addition of oxaliplatin increases toxicity without having any significant impact on tumor sterilization.

Optimal timing of surgery remains unclear. Previous recommendations to resect at 6-8 wk after neoadjuvant chemoradiation appear reasonable; but a longer interval may increase tumor downsizing. However, the effect of a longer waiting time on long-term outcome is yet to be defined.

The accuracy of studies attempting to determine tumor response remains problematic. Clinical complete response, as determined by existing methods, may not be equivalent to pCR. At this time, surgery remains the standard of care for rectal cancer patients following neoadjuvant chemoradiation. Observational management is still investigational but may be used in carefully selected high-risk patients, ideally in the setting of a clinical trial. Pathologic features such as ypT, ypN, tumor response, and CRM are accurate prognostic factors associated with long-term outcome.

Extrapolating from colon cancer trials, adjuvant chemotherapy (5FU/leucovorin, capecitabine or FOLFOX) remains the standard of care following rectal cancer resection, irrespective of pathologic response. Future directions will require a rethinking of management strategies, and may include different optimal drug combinations, treatment sequences, and approaches to neoadjuvant radiation, chemotherapy and/or targeted therapy. The goal is to achieve improvement in response and patient survival. The challenges include individualizing care to improve long-term oncologic outcome, while minimizing toxicity and maintaining quality of life.

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REVIEW

Experimental and clinical evidence of antioxidant therapy in acute pancreatitis

Mukaddes Esrefoglu

Mukaddes Esrefoglu, Department of Histology and Embryology, Bezmialem Vakif University Medical Faculty, 34093 Fatih, Istanbul, Turkey
Author contributions: Esrefoglu M wrote this paper.
Correspondence to: Dr. Mukades Esrefoglu, MD, Professor, Department of Histology and Embryology, Bezmialem Vakif University Medical Faculty, 34093 Fatih, Istanbul, Turkey. drmukaddes@hotmail.com
Telephone: +90-532-3465239 Fax: +90-212-5332326
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Abstract

Oxidative stress has been shown to play an important role in the pathogenesis of acute pancreatitis (AP). Antioxidants, alone or in combination with conventional therapy, should improve oxidative-stress-induced organ damage and therefore accelerate the rate of recovery. In recent years, substantial amounts of data about the efficiency of antioxidants against oxidative damage have been obtained from experiments with rodents. Some of these antioxidants have been found beneficial in the treatment of AP in humans; however, at present there is insufficient clinical data to support the benefits of antioxidants, alone or in combination with conventional therapy, in the management of AP in humans. Conflicting results obtained from experimental animals and humans may represent distinct pathophysiological mechanisms mediating tissue injury in different species. Further detailed studies should be done to clarify the exact mechanisms of tissue injury in human AP. Herein I tried to review the existing experimental and clinical studies on AP in order to determine the efficiency of antioxidants. The use of antioxidant enriched nutrition is a potential direction of clinical research in AP given the lack of clues about the efficiency and safety of antioxidant usage in patients with AP.

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Key words: Acute pancreatitis; Antioxidants; Clinical; Experimental; Oxidative stress

Peer reviewers: Dr. Andrzej Dabrowski, MD, PhD, Professor, Department of Gastroenterology and Internal Medicine, Medical University of Bialystok, ul. M. Sklodowska-Curie 24A, 15-276 Bialystok, Poland; Shiu-Ming Kuo, MD, University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo, NY 14214, United States

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INTRODUCTION

Acute pancreatitis (AP) can present as a wide clinical spectrum ranging from a mild, self-limiting localized disease to fatal widespread multi-organ failure with high mortality rates. Mild pancreatitis is the inflammation and edema of the pancreas, with additional features of necrosis and secondary injury to extrapancreatic organs in severe pancreatitis[1-3]. AP is characterized by acute inflammation and necrosis of pancreatic parenchyma, necrosis of pancreatic fat, hemorrhage, and inflammatory infiltration[4]. Since pancreatic cell death occurs according to either necrosis or apoptosis mechanisms, many necrotic and apoptotic cells can be seen within pancreas parenchyma. The study of Booth *et al*[5] found that oxygen free radical (OFR) induction in acinar cells promoted apoptosis whereas inhibition of OFR generation led to an increase in necrosis accompanied by reduced ATP. These findings suggest that OFR generation within acinar cells may be a protective response during pancreatitis. The main reason for necrotic or apoptotic cell death is the early activation of pancreas zymogens, especially cathepsin and trypsinogen inside the pancreas. Scott *et al*[6] showed that excessive OFR in

a pathologic state can cause tissue and cell damage. The OFR participates in the pancreas edema process in AP, and may participate in the pancreas necrosis process. Furthermore, OFR are involved in the generation of pain as another important clinical feature of patients suffering from AP[7]. The OFR, as highly reactive species, directly attacks lipids and proteins in the biological membranes and thus disrupts their functions. The action of OFR includes oxidation of lipids in the pancreatic cell membrane and oxidatively modified proteins, depolarization of the mitochondrial membrane, and induction of DNA fragmentation[8].

Aerobic organisms require ground state oxygen to live; however, the use of oxygen during normal metabolism produces OFR[9,10]. OFR are required for the maintenance of tissue homeostasis. Physiologic levels of OFR can regulate transcription, serve as signal molecules and defend against pathogen infections[11]. The mitochondria, endoplasmic reticulum, cytosol, and nuclear membranes have all been shown to be sources of OFR[12]. The primary function of the electron transport chain located in the inner mitochondrial membrane is ATP synthesis via oxidative phosporylation[13]. The superoxide radical formed during cellular metabolism is mainly produced during electron transport in the mitochondria and is released to both the mitochondrial matrix and intermembrane space[9,10,14,15]. Superoxide, depending on its location, causes potential oxidative damage to different proteins and lipids, as well as DNA[16]. The end product of the respiratory chain is water generated in a four-electron reduction of molecular oxygen. However, a small portion of oxygen is involved in the generation of OFR, including superoxide anion radicals, hydrogen peroxide, hypochlorous acid, and the extremely reactive hydroxyl radical[10,17]. These free radicals, each containing an unpaired electron, are energetically unstable and highly reactive. OFR can attack the double bonds of unsaturated phospholipids in cell membranes, which eventually degrade the structural integrity of cell membranes. Lipid oxidation can lead to the loss of the integrity of both plasma membrane and intracellular membranes, such as that of lysozomes and the endoplasmic reticulum, which then leads to an intracellular leak of proteases or an influx of Ca2+ resulting in necrosis[18]. OFR also impairs the functions of enzymes by causing fragmentation of polypeptide chains or cross-linking sulfhydryl groups in proteins. In addition, they cause strand breaks or abnormal cross-linking in DNA[19-21]. Damage to DNA may lead to a DNA-damage response, including activation of p53 and poly-ADP ribose polymerase (PARP) (a nuclear enzyme). While activation of p53 causes apoptosis and cell cycle arrest, hyperactivation of PARP leads to necrosis[22]. The hydroxyl radical is the most reactive species and can attack and damage almost all molecules found in the living cell. Being so reactive, it attacks DNA, a free radical chain reaction cascades through the DNA and causes chemical alterations of the bases as well as strand breakage. The best characterized biological damage caused by hydroxyl radicals may be their ability to stimulate the free radical chain reaction known as lipid peroxidation[12,23]. One hydroxyl radical can result in conversion of many hundred fatty acid chains into lipid hydroperoxides. Accumulation of lipid hydroperoxides in a membrane disrupts its functioning. Some of the products of lipid peroxidation, malonaldehyde (MDA) and 4-hydroxynonenal, increase the permeability and deformability of the membranes in which they are found. Mitochondrial membranes are particularly susceptible to this sort of damage, perhaps because they combine a high risk of OFR-mediated peroxidation[12]. We have shown mitochondrial degeneration within pancreatic acinar cells in experimental pancreatitis[1,24].

OFR indirectly act on the arachidonic acid cascade by increasing the production of thromboxane, which lowers tissue circulation by its potent platelet-aggregating and vasoconstricting effects[25]. Additionally, OFR enhance the production of leukotriene B4 which promotes activation of leukocytes and discharge of lysosomal enzymes[26]. As a secondary effect, polymorphonuclear leukocytes are responsible for the respiratory burst that leads to an enhanced production of radical species and activated enzymes and further cell damage[27]. The results of Rau *et al*[28] indicate that OFR play an important mediator function in early and later courses of AP. Their findings suggest that OFR species are important mediators but not necessarily triggers of tissue damage in AP. The degree of oxidant-antioxidant balance changes in the early phase of human AP, correlating with the clinical severity of pancreatitis[29]. Park *et al*[30] reported higher plasma levels of lipid peroxides and myeloperoxides and lower superoxide dismutase (SOD) activity in patients with severe AP than in those with mild AP. OFR may thus be closely associated with the inflammatory process and the severity of AP. Thareja *et al*[31] reported that high oxidative stress was observed during the early phase of AP and that gradually improving antioxidant status was associated with a better clinical outcome in patients with AP. The concentration of plasma lipid peroxides is a particularly meaningful index for determining the severity of the disease in humans[30,32].

During the later stages of AP, the major pathophysiological role seems to be the attraction and activation of leukocytes, which in turn contribute to enhanced radical generation and acinar cell damage[33,34]. Oxidative stress in the neutrophils (activated during the inflammatory response to acinar injury) may be responsible for further propagation of local and systemic inflammation[5]. Synergy between pro-inflammatory cytokines and oxidative stress occurs in the development of the inflammatory response in AP[35]. Proinflammatory cytokines such as interleukin (IL)-1, 6 and tumor necrosis factor (TNF) and microvascular ischemia are also important factors in the pathophysiology of AP[33,34]. Serum levels of proinflammatory cytokines, such as TNF-alpha and IL-1beta, increase during the course of AP and appear to be the driving force for the initiation and propagation of

the systemic response. Accordingly, pretreatment with either an antibody against TNF-alpha or a blockade of TNF-alpha production with pentoxifylline ameliorates experimental AP[36]. A cross-talk between oxidative stress and proinflammatory cytokines, particularly TNF-alpha amplifies the inflammatory cascade through different mechanisms, such as the activation of mitogen activated protein kinases and nuclear factor-kappa B (NF-κB) and/or the inactivation of protein phosphatases[35,37-39].

In addition to increased levels of plasma and tissue lipid peroxides, decreased levels of plasma antioxidants such as vitamin A, vitamin C[40], vitamin E[41], selenium[42], β carotene, whole-blood glutathione, and the activity of plasma glutathione peroxidase[21] have been found in patients with AP. Moreover, lower levels of selenium in toenails of AP patients and lower selenium concentrations in red blood cells of patients with severe AP have been detected[40].

Since experimental[42,43] and clinical studies[31,44] have provided some support for the concept that oxidative stress is the common pathway for the pathogenesis of AP, one reasonable idea is to use antioxidant regimens in the management of AP to complement its traditional therapy. In fact elucidation of all of the mechanisms above mentioned and their interactions is critical in developing a treatment based on the pathophysiology of AP. However, clinical evidence showed that antioxidant administration to patients with AP was not as beneficial as the antioxidant administration to experimental animals. Perhaps the key steps in the pathogenesis of AP are not yet fully understood. It is not possible to clearly determine whether oxidative stress is a cause or an effect of AP. This review highlights the experimental and clinical evidence of the benefit of various antioxidants in AP.

ANTIOXIDANT THERAPY IN AP

Antioxidants may function to prevent the formation of, or to detoxify, free radicals or to scavenge OFR. Halliwell[45] has proposed this useful definition, "an antioxidant is any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". In experimental pancreatitis, the beneficial effects of antioxidants may be associated with the inhibition of NF-κB activity[46]. Types of antioxidants include antioxidant vitamins (e.g., ascorbic acid, α tocopherol, β-carotene), inorganic antioxidants (e.g., selenium), synthetic antioxidants (e.g., butylated hydroxyanisole), and a range of plant-derived polyphenols[47]. Organisms widely use glutathione peroxidase, glutathione transferase, SOD, catalase (CAT), and a variety of other antioxidants to protect themselves against generation of OFR[48-50]. Some authors have studied the effects of various antioxidants in experimental animals and patients with AP. They used both natural and synthetic antioxidants in order to protect the pancreas from deleterious effects of OFR. The following sections summarize data obtained from experimental and clinical studies about the efficacy of some oxidants in AP.

Alpha tocopherol

Tocopherols belong to a class of phenolic antioxidants which can inhibit lipid auto-oxidation by scavenging free radicals and by reacting with singlet oxygen. The vitamin E activity of alpha-tocopherol may be attributed to its efficient inhibition of *in vivo* lipid oxidation[51]. Fat-soluble antioxidants act directly in the lipid bilayer of plasma and cell membranes by interacting with membrane lipophilic components. A natural antioxidant, alpha-tocopherol has been found to be beneficial in inhibiting intermolecular connections of lipid peroxides in liver of dogs with AP[52]. Vitamin E, including tocopherols and tocotrienols, is a fat-soluble antioxidant. To my knowledge the effect of vitamin E on AP has not been studied. Since they accumulate within tissues, fat-soluble substances have high toxic risk thereby limiting their clinicical application and widespread utilization. The results of combined therapies, including vitamin E, will be discussed below.

Ascorbic acid

Ascorbic acid functions in multiple, complex ways, acting as a hydrogen donor, a metal inactivator, and a peroxide destroyer[31]. The study of Bonham *et al*[53] demonstrated that plasma ascorbic acid concentration was significantly below normal in patients with early phase AP; however, Sajewicz *et al*[32] reported that patients with AP had double the plasma ascorbic acid values than healthy volunteers. Few studies have investigated the therapeutic efficacy of ascorbic acid in experimental animals with AP whereas many have examined its effects singly or within an antioxidant mixture in patients with AP. Two decades ago, Nonaka *et al*[54] reported that CV3611, a synthetic free radical scavenger prepared from ascorbic acid, had an important therapeutic effect on the development of AP in mice. However since then, another experimental or clinical study evaluating the benefit of this agent in AP has not been performed. Du *et al*[55] have reported that high dose vitamin C has a therapeutic effect in humans with AP. Their results indicate that vitamin C decreases hospitalization and duration of disease, and increases the cure rate by blocking lipid peroxidation, diminishing proinflammatory cytokines, and improving cellular immune function. The results of combination therapies will be discussed below.

Beta-carotene

Beta-carotene protects lipids by interfering with photosensitized oxidation, and behaves as a reducing agent by trapping radicals. In addition to its singlet oxygen-quenching properties, beta-carotene has good radical-trapping properties at low partial pressures of oxygen, a condition which prevails in healthy tissues. In biological systems, alpha-tocopherol and beta-carotene exhibit synergism by mutually reinforcing their respective activities.

Synergism also takes place in a cascade where ascorbic acid can be regenerated at the expense of more oxidizable substrates[51]. In patients with mild AP, the concentrations of beta-carotene at final review has been found significantly higher than those in patients with severe AP[56]. The correlation between low antioxidant level and high severity of disease suggests the utility of antioxidant supplementation therapies. Lavy *et al*[57] have reported some possible protective effects of treatment with beta carotene in regards to the severity of post-endoscopic retrograde cholangiopancreatography pancreatitis (ERCP). In a double-blind trial, 321 patients were given a single dose of natural beta carotene. The rate of severe pancreatitis was found to be lower in the beta carotene-treated group. Adverse events were not reported.

Caffeic acid phenethyl ester

Caffeic acid phenethyl ester (CAPE) is a phenolic compound and an active substrate of propolis. Several investigators have shown that CAPE acts as an anti-inflammatory by inhibiting the release of arachidonic acid from cell membranes, and suppressing cyclooxygenase (COX)-1 and COX-2 enzyme activities[58], and reducing antioxidant activity by lipoxygenase inhibition[59,60]. Buyukberber *et al*[46] have found CAPE to be benefical in improving the biochemical and histopathological findings in cerulein-induced AP in rats. Turkyilmaz *et al*[61] have also reported the beneficial effect of CAPE on acute necrotizing pancreatitis in rats. To my knowledge, no clinical data has been reported about the effect of CAPE on AP in humans. The data obtained from experimental animals are promising. CAPE has been shown to inhibit the production of proinflammatory cytokines by inhibiting nuclear transcription factor activity[62].

Carnitine

As an antioxidant, acetyl l-carnitine culminate most probably protects tissues from oxidative stress by stabilizing cell membranes, rendering them more resistant to free radicals, perhaps by facilitating the repair of the phospholipid bilayer damaged by oxidant stress, rather than acting as a direct scavenger of free radicals or decreasing their generation[63]. Data so far obtained would suggest that prior administration of acetyl l-carnitine ahead of caerulein challenge has proven protective efficacy that could possibly be ascribed, in part, to its regulation of the oxidant/antioxidant balance and modulation of nitric oxide (NO) release and myeloperoxidase activity that may ultimately lead to regulation of the inflammatory events associated with AP[64].

Green tea

Another naturally occurring antioxidant comes from the caffeine-free extract from leaves of green tea (Camellia sinensis), shown to reduce the degree of AP and attenuate the activation of the transcription factor NF-κB, as well as the formation of proinflammatory cytokines[65]. It also significantly decreases lipid peroxidation[65,66] and the formation of nitrogen-derived radicals in rodents[65].

Melatonin

Melatonin, the hormone produced mainly by the pineal gland, has been studied widely. Both *in vitro* and *in vivo* studies have identified melatonin as a potent scavenger of highly toxic hydroxyl radical and other oxygen-based radicals. Potent anti-inflammatory effects of melatonin may be related to a reduction of the inflammatory mediators produced during the inflammatory process[67]. Several experimental studies showed that melatonin is effective in reducing oxidative stress-mediated AP in rodents[68,69]. Recently, we reported a potent therapeutic effect of melatonin on caerulein-induced AP and associated liver injury[1]. We also demonstrated its potent effect on the protection of cell ultrastructure[43]. Melatonin decreases tissue MDA levels and increases antioxidant enzyme levels or activities[1,3,43]. Melatonin treatment was found to promote the spontaneous regeneration process of pancreatic tissue in rats[69]. Jaworek *et al*[70] have reported that pinealcetomy aggravates AP in the rat. Interestingly, Belyaev *et al*[71] presented the dynamic changes of endogenous melatonin in the early phase of human AP. Melatonin concentrations during the first 24 h after the onset of pain in younger patients (< 35 years) were significantly higher than levels in older patients (> 35 years). They concluded that high endogenous serum melatonin levels in the first 24 h after the onset of AP played a protective role and favoured a mild disease course in humans, especially in young patients. Another clinical study performed by Chen *et al*[72] indicates that delayed neutrophil apoptosis is associated with mild and severe AP in humans. Neutrophil apoptosis plays a critical role in minimizing the autodestructive potential of neutrophils. The data from the study show that melatonin promotes neutrophil apoptosis in human AP. Melatonin administration is promising for the treatment of AP in humans. However, further studies are still needed.

N-acetyl-cysteine

N-acetyl-cysteine (NAC) is a thiol compound which, by providing sulphydryl groups, can act both as a precursor of reduced glutathione and as a direct free scavenger, hence regulating the redox status in the cells. In this way, it can interfere with several signaling pathways that play a role in regulating apoptosis, angiogenesis, cell growth and arrest, and inflammatory response[73]. NAC has been found to be hugely effective in reducing oxidative stress-induced pancreatic injury in rats[74,75] by enhancing the ability of acinar cells to produce IL-10[74], preventing the impairment of Ca^{2+}[75], and downregulating the expression of chemokines, monocyte chemotactic protein-1, and macrophage inflammatory protein-2[76]. Moreover extrapancreatic complications (liver and lung injury) during AP induced by bile-pancreatic duct obstruction were palliated by NAC treatment[77]. Onur *et al*[78] have reported that NAC, especially combined with hyperbaric oxygen, decreases oxidative stress parameters, serum amylase, cal-

cium, and lactate dehydrogenase levels, as well as the histopathological score. To our knowledge, only one study shows the effects of NAC in patients with AP. Recently, Milewski *et al*[79] investigated the effects of NAC (administered by intravenous or oral route) on post-ERCP. Regretfully, NAC failed to demonstrate any significant preventive effect on post-ERCP pancreatitis or on serum and urine amylase activity. The results of combination therapies, including NAC, will be discussed below.

Resveratrol

Alcoholic AP predominates in countries where other forms of alcoholic drinks rather than wine are preferred. Presumably, wine contains factors that protect the pancreas against alcohol-induced AP. One of these factors may be resveratrol. Resveratrol, a naturally occurring antioxidant, acts as a free radical scavenger[80], but to date, no clinical study has been performed on antioxidant and free radical scavenger potentials of resveratrol on AP. Resveratrol has been found beneficial in AP in rats[81-83]. Lawinski *et al*[84] found stilbene derivatives (resveratrol and diethylstilbestrol) effective in preventing pancreatic cells from structural changes to OOH-induced AP in Wistar albino rats. Resveratrol and diethylstilbestrol protect the pancreas against prooxidative activity of hydroperoxide; stilbene derivatives significantly inhibit the free radical generating reactions. Leonard *et al*[85] have demonstrated that resveratrol can clear hydroxyl, superoxide, and metal inductive radicals. Kimura *et al*[86] have found that resveratrol inhibits lipoxygenase, an enzyme that is converted into powerful inflammatory and white cell stimulating agents known as leukotrienes, hepoxillins, and lipoxins through arachidonic acid. Resveratrol may exert its therapeutic effect on severe AP by lowering pancreatic OFR and reducing pancreatic tissue infiltration of neutrophils[87]. Due to its strong effect of inhibiting activation of NF-κB and reducing secondary activation of cytokines, resveratrol is regarded as a promising drug for blocking the initiation and progress of AP. Though no document as yet illustrates the function of resveratrol in AP in humans, primary trials have found that resveratrol could inhibit the production of TNF and IL-6 in pancreatitis. More research is required[87]. An increasing amount of data has confirmed that resveratrol could relieve the pathologic injury of the pancreas and extra-pancreatic organs (intestines, brain, lungs, *etc*.) induced by severe AP[88-90]. Further clinical studies are needed.

Quercetin

Quercetin is a naturally occurring plant flavonoid abundantly present in onions, fruits, and Chinese herbs. Several studies pointed out the beneficial biological activities of quercetin which include antioxidant, antiinflammatory, antiatherosclerotic, and anti-tumor properties[91-93]. The study of Carvalho *et al*[94] has demonstrated that the flavonoid quercetin attenuates the severity of cerulein-induced AP in mice. In particular, they reported that quercetin treatment reduces pancreatic inflammation and associated tissue injury through suppression of neutrophil infiltration, TNF-alpha, IL-1β and IL-6 cytokine production, and TNF-alpha expression, and increased IL-10 cytokine production. Since high oral and subcutaneous doses of quercetin did not manifest any clinical signs of toxicity, it may be safe and nontoxic in the treatment of AP; however, further experimental and clinical investigations are needed.

Selenium

Selenium, a trace element necessary for cellular function in many organisms, is a co-factor for the antioxidant enzyme glutathione peroxidase[95]. Glutathione peroxidase catalyses the reduction of both hydrogen peroxide and lipid hydroperoxides[96] and as such acts as an intracellular defence against free radical injury[97]. Nowadays, the role of micronutrients, in particular selenium, is receiving increased attention. Lower levels of selenium in toenails of AP patients and lower selenium concentrations in red blood cells of patients with severe AP have been found[40]. Selenium administration 24 h after induction of experimental AP has been demonstrated to be associated with amelioration of pancreatic injury and lung injury although it has not reversed the diminished serum selenium level[98]. Kuklinski *at al*[99] reported their clinical results of 4 years of selenium therapy. They concluded that an improvement in the prognosis of AP could be achieved if antioxidative selenium therapy with sodium selenite was introduced in time. In rare cases, total necroses and complications in organs only occurred in those patients who were admitted to this therapy too late. Wollschläger *et al*[100] reported that selenium therapy caused a significant increase in selenium, a moderate increase in glutathione peroxidase activity, and a significant decrease in MDA activity, while SOD remained unchanged in patients with AP. Lindner *et al*[101] did report, however, that substitution of sodium selenite had no beneficial effect of the clinical outcome of patients with AP (32 selenium administered, 35 placebo administered). On the other hand, Kocan *et al*[102] found selenium to be beneficial in decreasing inflammation in one patient with AP. The results of combination therapies including selenium will be discussed below.

RESULTS OF COMBINED ANTIOXIDANT THERAPIES

Recently we reported the beneficial effect of an antioxidant mixture of NAC and ascorbic acid on experimental AP in rats. The antioxidant mixture reduced tissue MDA levels and increased tissue CAT and glutathione peroxidase activities[1,42] in L-arginine-induced experimental AP. Hardman *et al*[103] have demonstrated that early exogenous anti-oxidant intravenous supplementation using a combination of NAC, selenium, and vitamin C reduces pancreatic injury in rats. However, a case-control study demonstrated no benefit from intravenous administration of these multicompound anti-oxidants in clinical

AP although critically, many patients in the clinical study received antioxidants relatively late in the course of the disease[104]. A randomized trial by Siriwardena *et al*[105] also failed to show any benefit to AP patients given selenium intravenously as part of a cocktail of antioxidants including NAC and vitamin C. On the other hand, Sateesh *et al*[106] demonstrated the beneficial effects of vitamin C and NAC on decreasing oxidative stress and improving antioxidant status in 23 patients with AP. They concluded that antioxidant supplementation associated with standard medical treatment may decrease the length of hospital stay and rates of complications in patients with AP, but a larger clinical trial is needed to support this hypothesis. The study of Bansal *et al*[107] showed that vitamin-based antioxidant therapy had no significant beneficial effect on organ dysfunction or on clinical outcomes in severe AP during the hospital stay. They administered vitamin A (intramuscularly), E (orally) and C (intravenously) together for a period of 14 d. Kuklinski *et al*[108] showed the beneficial effect of an adjuvant antioxidant therapy with selenium and D-alpha-tocopherol in 90 patients with necrotizing or mild AP. They reported that the average lethality rate of 34% fell to 1.1%. This study represents the vital benefits of antioxidant therapy. Uden *et al*[109] administered organic selenium, beta carotene, vitamin C, vitamin E and methionine to patients with recurrent pancreatitis (idiopathic chronic 8, alcoholic chronic 7, and idiopathic acute 5) and they reported the beneficial role of antioxidants in pain reduction. Recently, Zhao *et al*[110] reported the significant beneficial effects of the combination of ebselen and ethylhydroxyethyl cellulose (EHEC) on severe AP in Sprague Dawley rats. The mixture prevented pancreatitis- induced multiple organ injury. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H-one) is a non-toxic selenoorganic drug with anti-inflammatory, antiatherosclerotic, and cytoprotective properties that downregulates the production of OFRs[111,112]. Ebselen is a mimic of glutathione peroxidase that also reacts with peroxynitrite and can inhibit enzymes such as lipoxygenases, NO synthases, nicotinamide adenine dinucleotide phosphate oxidase, protein kinase C and H(+)/K(+)-ATPase[113,114]. The combination of ebselen and EHEC may be a new potential for treatment of severe AP[110].

While the trial clearly showed that the given combination of antioxidants was not effective in patients with AP when administered intravenously, antioxidants administered through another route may be beneficial, given that the benefits of enteral versus parenteral nutrition in patients with AP are well proven[115,116]. In particular, a growing body of clinical evidence from other disease settings suggests that supplementation of enteral nutrition with antioxidants can be beneficial[117,118].

In conclusion, based on the reported experimental studies, using antioxidant regimens in the management of AP as a supplement and combined with traditional therapy is rational and reasonable. If this hypothesis is correct, antioxidant therapy should reduce the inflammatory process involved in pancreatitis and thereby accelerate the recovery rate. Some of the antioxidants have been shown to be beneficial in the treatment of human AP. However, the present studies indicate that insufficient clinical data support using antioxidants alone or in combination with conventional therapy in the management of AP. Further double blind, randomized, placebo-controlled clinical trials with a larger sample size need to be conducted.

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ORIGINAL ARTICLE

Dietary copper triggers onset of fulminant hepatitis in the Long-Evans cinnamon rat model

Ramsi Siaj, Vanessa Sauer, Sandra Stöppeler, Hans-Ullrich Spiegel, Gabriele Köhler, Andree Zibert, Hartmut HJ Schmidt

Ramsi Siaj, Vanessa Sauer, Andree Zibert, Hartmut HJ Schmidt, Klinik und Poliklinik für Transplantationsmedizin, Universitätsklinikum Münster, D-48149 Münster, Germany
Sandra Stöppeler, Hans-Ullrich Spiegel, Abteilung Chirurgische Forschung, Klinik und Poliklinik für Allgemein- und Viszeralchirurgie, Universitätsklinikum Münster, D-48149 Münster, Germany
Gabriele Köhler, Gerhard-Domagk-Institut für Pathologie, Universitätsklinikum Münster, D-48149 Münster, Germany
Author contributions: Siaj R, Sauer V and Stöppeler S performed the work; Siaj R drafted the manuscript; Spiegel HU and Köhler G analyzed data; Zibert A and Schmidt HHJ contributed to conception and design, and revised the manuscript.
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Correspondence to: Hartmut HJ Schmidt, MD, Klinik und Poliklinik für Transplantationsmedizin, Universitätsklinikum Münster, Albert-Schweitzer-Campus 1, Gebäude A14, D-48149 Münster, Germany. hepar@ukmuenster.de
Telephone: +49-251-8357935 Fax: +49-251-8357771
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Abstract

AIM: To investigate the impact of dietary copper given at different time points on the onset of fulminant hepatitis.

METHODS: The Long-Evans cinnamon (LEC) rat model of Wilson's disease (WD) was used to study the impact of high dietary copper (hCu) on the induction of fulminant hepatitis at early or late time points of life. High Cu diet was started in rat pups or in adults (month 5) for three months. Animals that received reduced dietary copper (rCu) throughout their lifetime served as a control. Hepatitis-associated serum markers (alanine aminotransferase, aspartate transaminase, bilirubin) were analyzed in animal groups receiving hCu or rCu. Liver copper content and liver histology were revealed at sacrifice. A set of 5 marker genes previously found to be affected in injured liver and which are related to angiogenesis (*Vegfa*), fat metabolism (*Srebf1*), extracellular matrix (*Timp1*), oxidative stress (*Hmox1*), and the cell cycle (*Cdkn1a*) were analyzed by real-time polymerase chain reaction.

RESULTS: Regardless of the time point when hCu was started, LEC rats (35/36) developed fulminant hepatitis and died. Animals receiving rCu (36/36) remained healthy, did not develop hepatitis, and survived long term without symptoms of overt disease, although liver copper accumulated in adult animals (477 ± 75 μg/g). With regard to start of hCu, onset of fulminant hepatitis was significantly ($P < 0.001$) earlier in adults (35 ± 9 d) that showed pre-accumulation of liver copper as compared to the pup group (77 ± 15 d). Hepatitis-associated serum markers, liver copper and liver histology, as well as gene expression, were affected in LEC rats receiving hCu. However, except for early and rapid onset of hepatitis, biochemical and molecular markers were similar at the early and late time points of disease.

CONCLUSION: Rapid onset of fulminant hepatitis in asymptomatic LEC rats with elevated liver copper suggests that there is a critical threshold of liver copper which is important to trigger the course of WD.

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Key words: Wilson's disease; Fulminant hepatitis; Acute liver failure; Dietary copper; Long-Evans cinnamon rat; *ATP7B*

Peer reviewer: Yuan-Ping Han, Assistant Professor, Surgery and Pathology, University of Southern California, 2011 Zonal Ave HMR 813, Los Angeles, CA 91780, United States

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INTRODUCTION

Wilson's disease (WD) is a genetic disorder transmitted by a recessive gene located on chromosome 13[1,2]. Diagnosis of WD is mostly established on the basis of combined biochemical and clinical parameters, and lately by specific genetic analysis[3-6]. Presentation of WD includes hepatic insufficiency, acute and chronic active hepatitis, fulminant hepatitis and/or extra hepatic manifestations. Although it is a rare disease, fulminant hepatitis is frequently observed in WD patients and is followed by high mortality that accounts for approximately 5% of acute liver failure (ALF) observed worldwide. The disease is linked to an imbalance of copper homeostasis that is due to mutation of the *ATP7B* copper transporter gene expressed primarily in the liver[7,8]. More than 600 mutations have been reported in human *ATP7B*. Effective drug treatment of WD has been established which aims at chelation or reduction of copper[1]. However, compliance to take lifelong treatment or to reduce dietary copper intake is low in patients[9]. Liver transplantation remains the only treatment option of WD patients encountering ALF.

In healthy individuals copper is absorbed *via* the intestine and delivered to the liver, where it is either transferred by *ATP7B* into ceruloplasmin that is secreted into blood or exported *via* bile[2]. Malfunction of the *ATP7B* gene in WD patients leads to large quantities of toxic copper in the liver and other organs. However, manifestation and onset of the disease induced by toxic copper can vary to a great extent between WD patients[10-12]. Individual mutations of the *ATP7B* gene as well as mutations outside of this locus have been implicated to be important for the course of the disease as suggested, e.g., by rare studies of twins having the same mutation of the *ATP7B* gene but presenting different courses of WD[13,14]. Besides genetic factors, environmental factors, such as the amount of dietary copper intake, may also have an impact on WD[15,16]. However, studies of such factors are difficult in humans.

Much has been learned from animal models of WD. The Long-Evans cinnamon (LEC) rat has a large deletion of the *ATP7B* gene[17] and has many characteristics that are also observed in patients, e.g., low ceruloplasmin, high liver copper levels, and sensitivity to anti-copper therapy. The LEC rat strain can be housed on a commercial diet containing standard copper concentrations (about 7-15 mg/kg). Using this standard chow, 40%-60% of LEC rats spontaneously encounter fulminant hepatitis at the age of 80-120 d[18]. The LEC rat is therefore a highly attractive model to analyze pathogenesis of the liver, and is also used to study the effect of novel therapeutic approaches for liver disease, e.g., hepatocyte transplantation or gene transfer[19,20].

In the present study, the role of low and high dietary copper given at different time points was investigated in male and female LEC rats. We hypothesized that preloading of copper may affect the course and onset of disease. A high copper (hCu) diet was given to juvenile and adult rats that could pre-accumulate liver copper. The impact on hepatitis, liver copper, histology, gene expression, and survival was examined.

MATERIALS AND METHODS

Animals

LEC rats that lack functional *ATP7B*[17] and Long-Evans agouti (LEA) rats expressing wild-type *ATP7B* were a kind gift of S. Gupta (Albert Einstein College of Medicine, New York, United States). Rats were genotyped for *ATP7B* by polymerase chain reaction (PCR) analysis using primers essentially as described previously[21]. For the hCu regimen, animals received a solid diet that contains a standard concentration (13 mg/kg) of copper (1324, Altromin, Germany). Animals of the hCu groups additionally received tap water containing 20 mg copper/L [copper (Ⅱ) chloride, Sigma-Aldrich]. For the reduced copper (rCu) regimen, rats received a specialized solid diet (C1041, Altromin) with a copper content of 0.3 mg/kg and distilled tap water. Female rats (n = 12) which were reported to have higher sensitivity to copper[18,22] and male rats (n = 6) were analyzed for each group. LEA rats, heterozygotes and LEC rats housed on standard diet were used as controls. The incidence of severe jaundice with strong disturbance of common as well as spontaneous behaviour, and weight loss (> 20%) which was accompanied by coma, led to sacrifice of the animals. The protocol for animal use was approved by local authorities.

Analysis of serum samples

Blood was taken under retrobulbar anesthesia with isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, Abbott). Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and total concentration of bilirubin in serum were analyzed photometrically using a Cobas Modular System (Roche Diagnostics, Germany). Ceruloplasmin oxidase activity was determined using the modified protocol of Schosinsky *et al*[23] adapted to a 96-well plate.

Liver histology

For histological evaluation, liver specimens were fixed by immersion for at least 24 h in 4% formaldehyde solution, and were subsequently dehydrated and embedded in paraffin wax in order to cut serial sections at a thickness of 5 μm. Morphologic parameters were determined after hematoxylin and eosin staining using standard protocols, and scored by a blinded observer, e.g., for polyploidy, steatosis, apoptosis, and proliferation.

Table 1 Genes analyzed by real-time polymerase chain reaction in liver

Gene symbol	Synonym	Accession number	Primer (forward/reverse)
Vegfa	Vascular endothelial growth factor A	NM031836	CAGTTCGAGGAAAGGGAAAGGCAAATGCTTTCTCCGCTCTG
Hmox1	Heme oxygenase (decycling) 1	NM012580	AGAGGCTAAGACCGCCTTCC AGGCCTCTGGCGAAGAAAC
Cdkn1a	Cyclin-dependent kinase inhibitor 1A	U24174	CTTGCACTCTGGTGTCTCACG ATCGGCGCTTGGAGTGATAG
Timp1	TIMP metallopeptidase inhibitor 1	BC099821	CCTGGTTCCCTGGCATAATC TTGCAAGGGATGGCTGAAC
Srebf1	Sterol regulatory element binding protein-1	AF286470	AGAAGGCCAGTGGGTACCTG TGCGGGCCACAAGAAGTAG

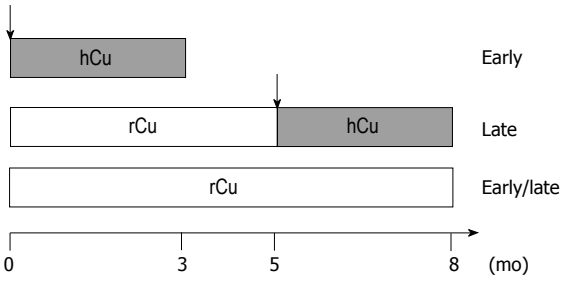


Figure 1 Schematic representation of study design. For the early copper regimen, Long-Evans cinnamon (LEC) rat pups received a high copper (hCu) diet up to month 3. For the late copper regimen, LEC pups were first housed on a reduced copper (rCu) diet until adult (month 5). Thereafter, rats received hCu for 3 mo up to the end of observation time (month 8). LEC rats received rCu diet throughout the observation period (early/late). *ATP7B* heterozygotes as well as Long-Evans agouti rats were also studied using an early hCu regimen. Arrow indicates start of hCu regimen.

Copper determination

For determination of copper content, liver samples were dried for 72 h at 70 ℃. Dry weight was determined using the analytical balance ME235S (Sartorius, Germany). Liver was redissolved in 65% nitric acid (Merck, Germany) and copper concentration was determined by flame atomic absorption spectroscopy (Shimadzu AA6300, Japan).

Real-time PCR analysis

Randomly chosen liver samples were immediately frozen after resection. Total RNA was extracted after disruption of tissue using a homogenizer and RNeasy kit (Qiagen, Hilden, Germany). Reverse transcription-PCR was carried out as described[24]. Briefly, RNA was reverse transcribed using SuperScript Ⅱ according to the instructions of the manufacturer (Invitrogen). Primers for PCR (Table 1) were synthesized by MWG Biotech (Germany). For quantitative real-time PCR, a SYBR® Green kit (Eurogentec, Belgium) was used. PCR was performed on the ABI PRISM™ 7900HT sequence detector (PE Applied Biosystems, United States). Each sample was tested in three independent experiments. The ct value was normalized to the expression of the house-keeping gene *HPRT* (Δct method). Relative expression level of gene normalized to house-keeping gene was determined by the equation $2^{\Delta ct}$ and fold-change was calculated thereafter.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Data are given as mean ± SE and median. Data were analyzed by the Student's two tailed *t* test using Bonferroni correction for post-hoc pairwise comparisons or Kruskal-Wallis test. Kaplan-Meier survival test and logrank test were performed for time to event data.

RESULTS

Onset of fulminant hepatitis

The role of low and high dietary copper in the induction of severe liver disease was studied with respect to the start of a copper diet in LEC rats of different age (Figure 1). A hCu was started early in pups or late in adults at month 5. The latter animals received a rCu up to month 5. The time point of hCu in adults was chosen to encompass the phase of fulminant hepatitis that occurs in LEC rats at the age of 80-120 d when using commercial chow[18]. LEC rats subjected to hCu displayed significantly elevated hepatitis-associated markers (Figure 2A), and developed severe jaundice (bilirubin > 2.0 mg/dL). With regard to start of hCu, levels of serum markers increased significantly earlier ($P < 0.001$) after the late regimen as compared with the early regimen (Figure 2B). Both genders displayed similar onset of significantly elevated serum markers (Table 2). LEC rats receiving a rCu diet or animals of the control groups did not develop hepatitis.

LEC rats (36/36) receiving rCu survived the observation period (8 mo), and no signs of overt morbidity were recorded in individual rats (18/18) as compared to controls, even when monitored beyond the observation period (up to month 20). In contrast, survival was significantly impaired ($P < 0.001$) in LEC rats (35/36) that received hCu (Figure 2C). LEC rats subjected to an early hCu regimen showed a mean survival of 77 ± 15 d that was only moderately shorter when compared to control LEC rats that were housed on a commonly used standard diet (84 ± 5 d). In contrast, LEC rats that received a late hCu regimen had a significantly reduced survival (35 ± 9 d) with regard to start of hCu. Survival of male and female LEC rats did not differ (Table 2).

Impact of dietary copper on liver histology

Nuclei of hepatocytes were found to be highly enlarged in LEC rats receiving early and late hCu regimens (Figure 3). Pronounced fatty acid changes, inflammation and, to a lesser extent, necrosis were observed in LEC rats receiving hCu. No marked changes in liver histology, including enlargements, fatty metamorphosis and inflammation, or cholangiofibrosis, that are routinely found in adults[18] were observed in LEC rats that received a rCu diet. The liver histology in the control groups was found to be

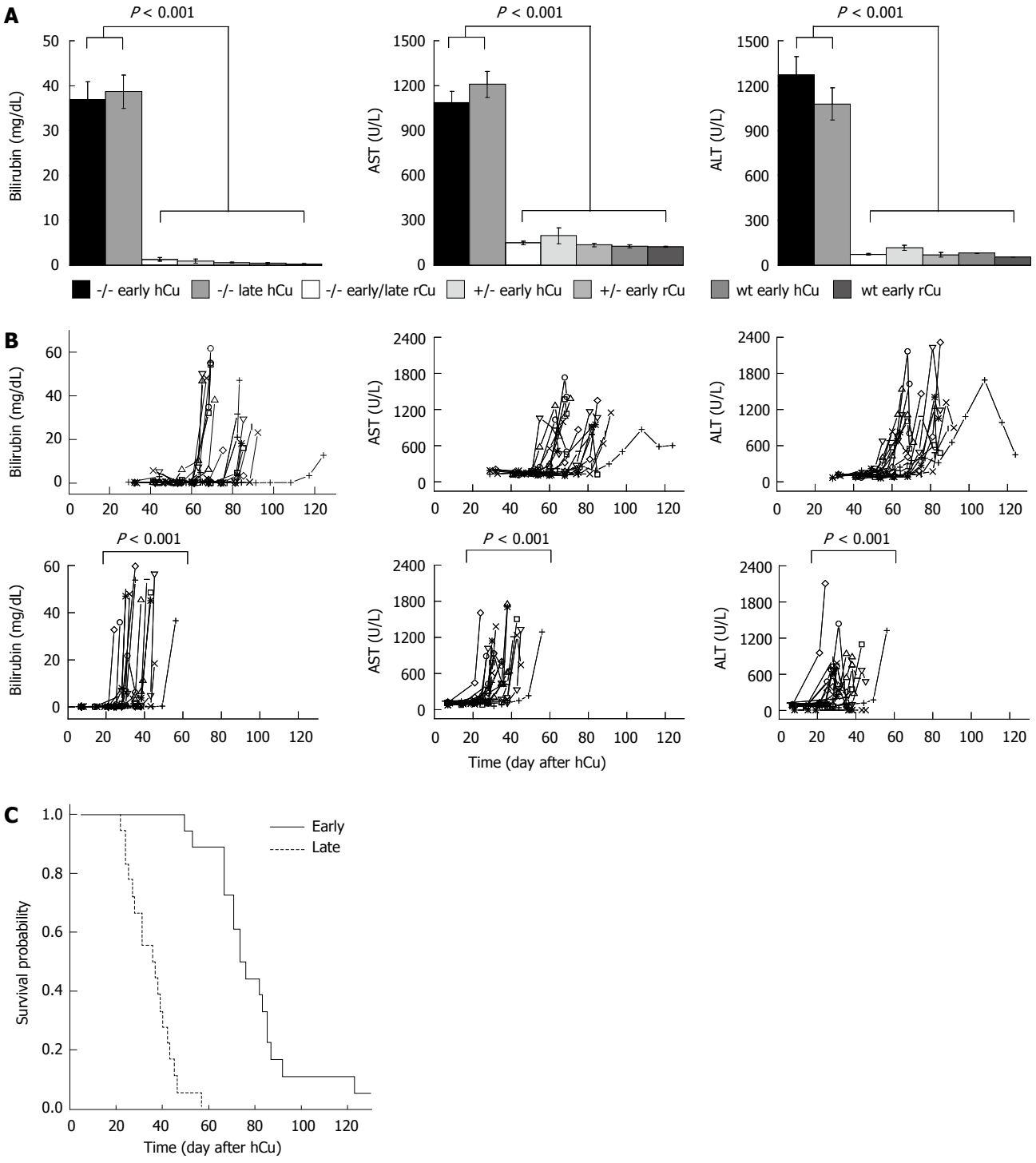


Figure 2 High copper regimen induces fulminant hepatitis. A: Bilirubin, aspartate transaminase (AST) and alanine aminotransferase (ALT) were determined in Long-Evans cinnamon (LEC) rats (-/-), heterozygotes (+/-) or Long-Evans agouti (LEA) rats (wt). Animals received a high copper (hCu) or reduced copper (rCu) diet as an early or late regimen. Median ± SE of maximal values obtained from animals are shown; B: Time course of serum markers in LEC rats that received early (top) or late (bottom) hCu. X-axis represents day after start of hCu. Animals of other groups showed normal levels. Of note, the onset of increased values following late hCu was at day 189 ± 42 after birth. Each line represents one animal. Logrank test using threshold (mean ± SE of LEA rats ×2) was used; C: Kaplan-Meier survival curve of LEC rats receiving an early and late hCu regimen are shown. Survival is represented as day after start of hCu. Of note, age of animals at death in late group was 190 ± 9 d.

normal regardless of the copper regimen used.

Accumulation of liver copper

High concentrations of copper were determined in the livers of LEC rats (Figure 4) following hCu, resembling WD[25]. LEC rats receiving rCu exhibited significant liver copper after the early (422 ± 62 μg/g) and late regimen (477 ± 75 μg/g) as compared to heterozygotes or wild type rats. Male and female LEC rats did not significantly differ with respect to liver copper (Table 2). Ceruloplasmin activity, which is absent in the LEC rat strain[20], was not affected by a copper diet (Figure 5).

Table 2 Male and female Long-Evans cinnamon rats after copper diet

		Early hCu		Late hCu		rCu		Long-Evans agouti
		Male	Female	Male	Female	Male	Female	Female
Bilirubin	> 0.4 mg/dL[1] (d)	55 (67 ± 5)	63 (60 ± 1)	28 (30 ± 1)	28 (32 ± 1)	0.3[2] (0.3 ± 0)	0.2[2] (0.2 ± 0)	0.2[2] (0.2 ± 0)
AST	> 240 U/L[1] (d)	74 (75 ± 3)	68 (68 ± 1)	28 (34 ± 3)	30 (33 ± 1)	115[2] (117 ± 4)	103[2] (103 ± 3)	114[2] (114 ± 5)
ALT	> 110 U/L[1] (d)	44 (50 ± 2)	54 (54 ± 1)	28 (30 ± 2)	28 (31 ± 1)	69[2] (69 ± 6)	75[2] (75 ± 6)	54[2] (54 ± 1)
Liver Cu	(μg/g)	868 (880 ± 93)	1036 (1058 ± 59)	1494 (1452 ± 161)	1235 (1178 ± 59)	503 (495 ± 75)	309 (326 ± 29)	46 (44 ± 9)
Survival	(day after hCu)	85 (81 ± 4)	81 (75 ± 9)	37 (37 ± 6)	35 (35 ± 2)	NA	NA	NA

NA: Not available; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; hCu: High copper; rCu: Reduced copper. [1]Sera up to sacrifice were analyzed by logrank test using thresholds calculated from Long-Evans agouti rat (mean ± SE) × 2; [2]Max values up to sacrifice are given. Data of male and females did not differ (logrank, *t* test, Kruskal-Wallis, Kaplan-Meier).

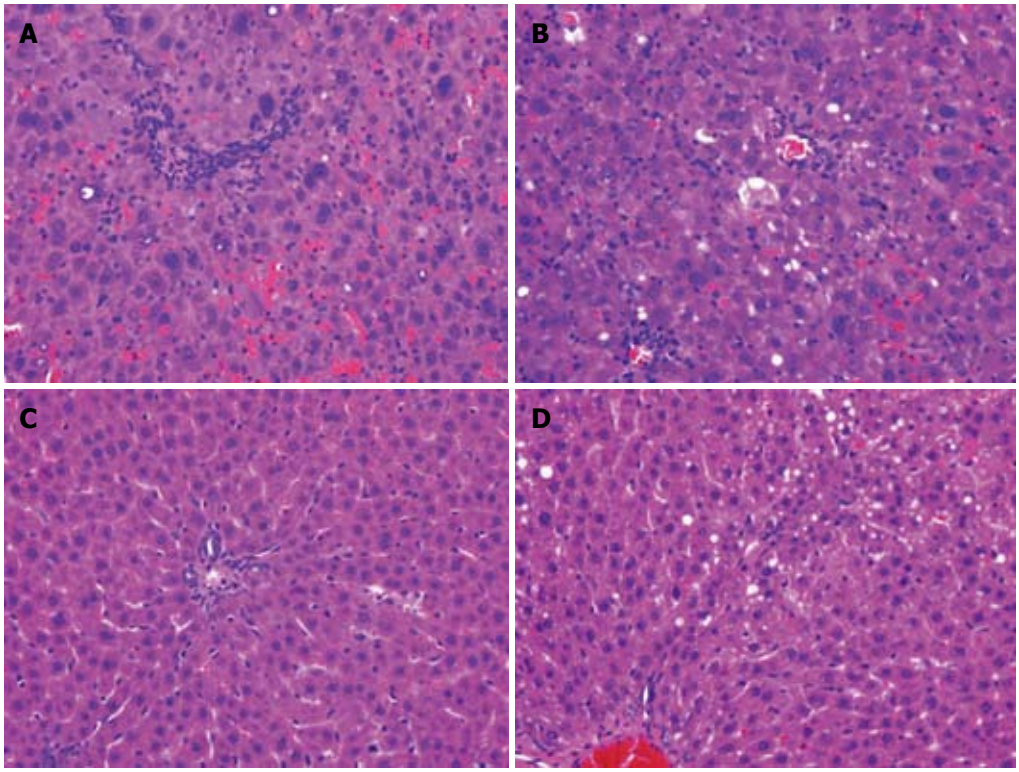


Figure 3 Liver of Long-Evans cinnamon rats is significantly affected by high copper regimen. Hematoxylin and eosin staining of liver. Livers were obtained from Long-Evans cinnamon rats that received high copper at an early or late time point (A, B) or after a reduced copper diet (C: late regimen). As a control, liver of Long-Evans agouti rat is depicted (D). Note highly irregular architecture of liver parenchyma, polyploidy, enlarged nuclei and inflammation (A, B) as compared to C and D. Liver histology of other groups was normal. One representative staining (40× magnification) of female rats is shown.

Gene expression in liver

In order to investigate whether the different copper diet regimens also have an impact on major biochemical pathways, gene expression in the liver was determined in animals at sacrifice. A small set of five marker genes was analyzed that relate to different areas of metabolism previously found to be affected in injured liver, including angiogenesis (*Vegfa*), fat metabolism (*Srebf1*), extracellular matrix (*Timp1*), oxidative stress (*Hmox1*), and the cell cycle (*Cdkn1a*). A highly different (fold-change > 8.5) expression of marker genes was observed when LEC rats receiving an early hCu regimen were compared to LEC rats receiving rCu (Figure 6). mRNA levels of Vegfa (8.5) and Srebf1 (13.9) were downregulated at the time points of fulminant hepatitis in LEC rats receiving hCu, whereas Timp1 (9.8), Cdkn1 (10.1) and Hmox1 (10.6) were upregulated at this time point. Marker gene expression was not affected in LEC rats receiving rCu, in LEA rats or in *ATP7B* heterozygotes (data not shown). Of note, none of the five genes displayed a significantly different (fold-change > 2) level of expression when LEC rats receiving early and late hCu regimen were compared.

DISCUSSION

The early hCu regimen used here for juvenile LEC rats closely resembles previous protocols. Onset of fulminant hepatitis was observed by us at about day 80, which is of a similar range as compared to others[18,25,26]. The high dietary copper regimen was achieved by copper

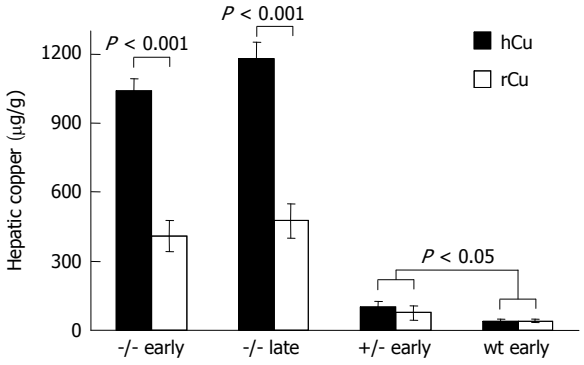


Figure 4 Increased liver copper is found in Long-Evans cinnamon rats irrespective of copper diet. Copper was determined in the liver of Long-Evans cinnamon rats (-/-; *n* = 9), heterozygotes (+/-; *n* = 5) and Long-Evans agouti rats (wt; *n* = 3) after receiving high copper (hCu) or reduced copper (rCu) diet according to an early or late regimen, respectively. Copper concentration is given as mean ± SE of liver dry weight.

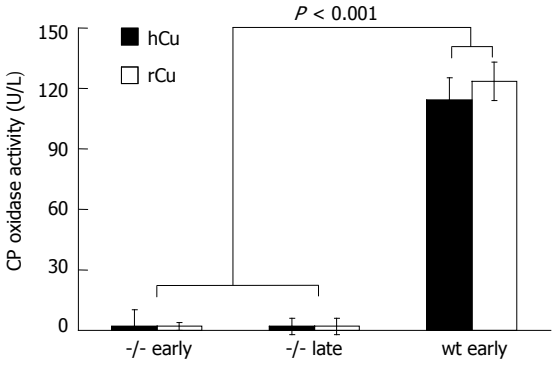


Figure 5 Ceruloplasmin oxidase is independent of high copper treatment. Ceruloplasmin (CP) oxidase was determined in Long-Evans cinnamon rats (-/-) that received high copper (hCu) or reduced copper (rCu). Sera (*n* = 6) were obtained from animals after an early or late hCu regimen. As control, values obtained from Long-Evans agouti rats (wt) (*n* = 3) are shown. Data are expressed as mean ± SE.

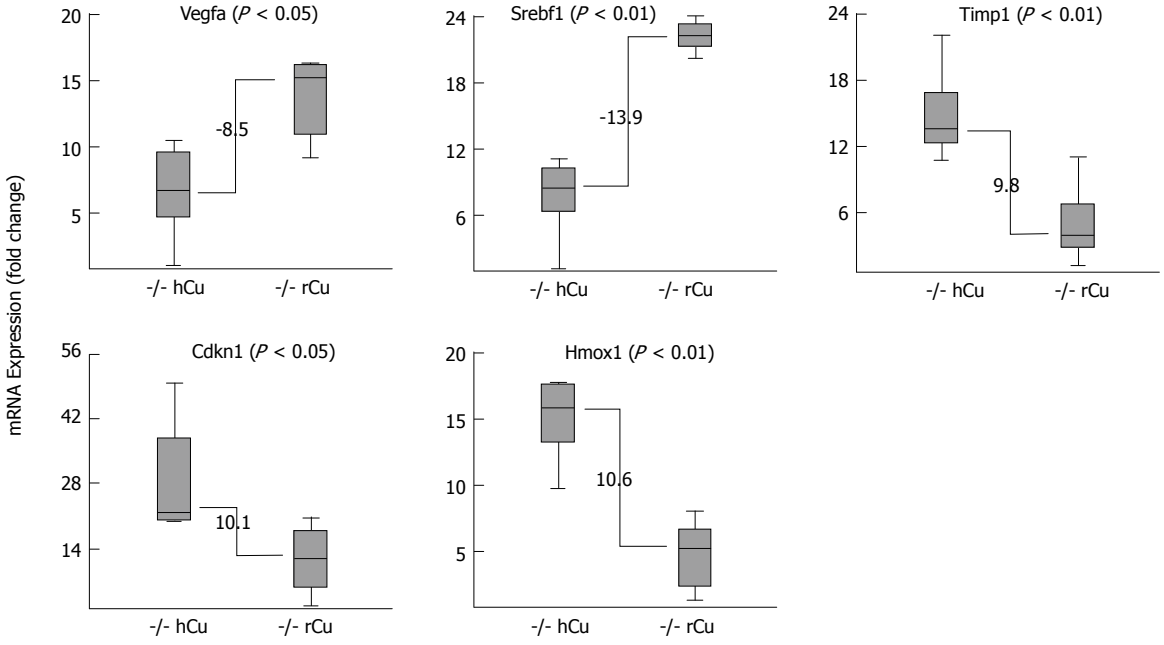


Figure 6 Liver gene expression of Long-Evans cinnamon rat is significantly altered after high copper regimen. Relative expression of individual mRNA in Long-Evans cinnamon (LEC) rats (*n* = 7) that were subjected to early high copper (hCu) regimen (left boxes) and in age-matched LEC rats (*n* = 5) receiving reduced copper (rCu) (right boxes). Animals were analyzed at fulminant hepatitis. Fold-change was calculated within groups by the Δct method using the *HPRT* gene for normalization. Data are shown as a box plot representation as calculated from three independent experiments. Numbers refer to difference of fold-change with regard to the median of each group.

enriched tap water (20 mg copper/L) and a standard solid diet (13 mg copper/kg). As compared to previous studies that employed a standard solid diet, only the overall dietary copper burden given to the LEC rats was moderately increased[18,25,26]. Much higher dietary copper concentrations have been reported in LEC rats[26-28], and intraperitoneal injection of 3 mg/kg copper for 3 d was shown to result in jaundice and fulminant hepatitis within 2 d after the last injection, suggesting that besides the absolute amount of copper the route of administration is also important[29]. As one result of our high dietary copper regimen, all LEC rats (with the exception of one animal used for the early regimen) encountered fulminant hepatitis and death. This is in contrast to previous studies using a standard solid diet only, where 40%-60% of LEC rats survived[18,25,26]. Since the high dietary copper regimen can induce fulminant hepatitis in nearly all LEC rats, such protocols may now allow a more quantitative analysis of therapy, e.g., by cell-based approaches[30].

Prevention of hepatitis and almost disease-free long term survival were observed in LEC rats that received a low copper diet throughout their lifetime. Hepatitis in LEC rats is closely associated with copper intake[18,31], and it was therefore noted early that a copper deficient diet can reduce serum levels of AST and ALT in addition to

diminution of copper concentration in the liver[27]. In the latter study of male LEC rats, animals at the age of 30 d were monitored for 35 d or 15 wk[18,32]. A rCu diet that was enriched for L-proline could also improve survival (up to month 5) in male LEC rats[26]. We could confirm previous results regarding the impact of reduced dietary copper intake in a long-term study (up to 20 mo). Since male LEC rats seem to be more resistant to copper[33,34], we also investigated female LEC rats; however, a significant difference between genders was not observed for onset of hepatitis, liver copper, and survival.

Onset of hepatitis as determined by serum markers ALT, AST, and bilirubin was significant earlier (about 2 wk) in LEC rats that received a late hCu regimen. The finding of a significantly earlier onset of hepatitis and death in animals having elevated liver copper at start of hCu suggests that copper preloading of the liver above a critical threshold may accelerate the onset of disease. In contrast, LEC rats remained apparently asymptomatic showing liver copper levels that were about 10-fold higher as compared to LEA rats, suggesting that a high liver copper level can be tolerated for an extended time but induces a rapid onset of disease when dietary copper intake is increased. It should be stated that the effect of copper preloading could not be demonstrated by inspection of liver tissue since the stains obtained at early and late time points after hCu treatment did not significantly differ. Inflammation and necrosis were almost absent in stains of liver tissue at early and late time points when a rCu diet was used, suggesting that preloading with significant but subcritical levels of copper does not result in gross histological alterations of liver tissue and adverse immune responses.

The liver of LEC rats was characterized by determination of mRNA levels after a hCu regimen to assess the impact of dietary copper on gene expression. Notably, an almost identical gene expression was observed in juvenile and adult LEC rats after the hCu regimen, indicating that pre-accumulated copper levels do not affect gene expression at least at the time point of fulminant hepatitis. Marked differences were however observed between LEC rats at fulminant hepatitis and age-matched asymptomatic LEC rats that received a rCu diet. Inhibitory molecule Cdkn1a (p21) was found to be markedly upregulated suggesting that the cell cycle is blocked at G0 in the phase of fulminant hepatitis, confirming previous results obtained in LEC rats as well as in rats after hepatectomy[35,36]. It is conceivable that arrest of the cell cycle within the liver is a common molecular determinant of fulminant hepatitis. Timp1, an important mediator of extracellular matrix remodeling during toxic liver injury[37], was highly upregulated in LEC rats after hCu corroborating our observations of significant changes in liver architecture. The role (if any) of Timp-1 mRNA regulation by a copper diet with regard to the activity of metalloproteinase (MMP), e.g., MMP-9, which was shown to be highly induced in fulminant hepatic failure[38], remains to be studied. Expression of *Srebf1* was down-regulated in LEC rats after the hCu regimen, suggesting that copper overload may be followed by reduction of lipid biosynthesis. Of note, Srebf1 activity was found to be increased in copper-deficient rats[39]. *Hmox1*, which is related to oxidative stress induced by iron deposition that is also commonly observed in LEC rats, was found to be highly upregulated after the hCu regimen, as reported before[36]. In contrast, *Vegfa*, a strong mediator of angiogenesis that was recently observed to be induced in hepatoma cells by hCu *via* a ceruloplasmin promotor, was down-regulated after hCu regimen. LEC rats which have a low ceruloplasmin expression may differ in this respect from wild type[40]. Although our analysis of gene expression in the liver of LEC rats after hCu regimen showed a significant modulation of important markers of copper metabolism, the study is limited since only one time point was examined. Clearly, further studies are needed that are beyond the scope of this article to explore the molecular events following toxic copper exposure of the liver.

Accumulation of dietary copper in the liver over a lifetime may play a role in the course of WD even when the genetic constitution of patients is similar[13-16]. While WD is frequently observed in the 2nd and 3rd decade of life, presentation of typical WD symptoms at the preschool age is relative rare and onset of disease above the age of 40 has been noted[12]. The finding that LEC rats have moderate liver copper but no overt disease may possibly resemble the observation of rare asymptomatic WD patients[41,42]. Conversion from a state of low to high dietary copper is followed by rapid development of severe disease in LEC rats, a situation that may also apply to WD patients after discontinuation of therapy[9,43]. Our findings indicate the importance of a stringent maintenance of anti-copper therapy for management of WD patients in order to balance liver copper level below a critical threshold, and reinforce the lifetime risk of WD patients of developing liver failure when dietary copper intake is increased or anti-copper therapy has failed.

Taken together our results demonstrate that asymptomatic LEC rats having significantly elevated liver copper either rapidly develop fulminant hepatitis when dietary copper is increased or survive long term on a rCu diet, suggesting that there may exist a critical threshold of liver copper important to trigger onset of WD. A stringent diet of low copper throughout the lifetime is followed in LEC rats by escape from hepatitis, moderately low liver copper, and long term survival without overt disease. The data underline the importance of environmental factors, such as dietary copper, with respect to the phenotype and course of WD, and suggest that similar molecular and physiologic mechanisms are initiated when tolerable copper thresholds are exceeded during the lifetime.

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COMMENTS

Background

Fulminant hepatitis is a life-threatening disorder that is also observed in Wilson's disease (WD), an inherited disease that is caused by mutation of the *ATP7B* gene encoding a crucial copper transport protein in the liver. The dietary intake of copper during a lifetime can affect the course of WD. Analysis of dietary copper intake was assessed in a rat model of WD. The study is related to the understanding of copper toxicity and the molecular events of pathogenesis.

Research frontiers

High copper is toxic to cells. In humans, the liver has the role of excreting excessive copper and preventing toxicity. The molecular events following copper toxicity are not understood. The relationship between liver copper levels and onset of disease is important to understand for improving current therapy approaches.

Innovations and breakthroughs

Accumulation of liver copper in individuals carrying two allelic mutations of *ATP7B* is ultimately followed by development of WD; however, rate, onset, course of disease, including acute liver failure (ALF), varies greatly between patients. Studies of monozygotic twins having identical mutations of *ATP7B* are most informative to decipher the role of genetic and environmental factors for the course of WD. However, these studies are casual and rare. The Long-Evans cinnamon rat carrying a natural mutation of *ATP7B* has been established as a model to study the pathophysiology of WD. This study illustrates for the first time that a stringent copper-restricted diet can completely prevent development of fulminant hepatitis in the long-term (up to 20 mo). Reduced dietary copper intake is associated with no overt disease symptoms, although elevated levels of liver copper were observed. This state of disease rapidly shifts to severe disease, liver failure, and death if dietary copper intake is increased. The molecular events following induction of ALF seem to be identical in WD regardless of the time point of disease onset.

Applications

The observation of different degrees of disease progression with regard to liver copper levels underlines the importance of a stringent maintenance of drugs during anti-copper therapy of WD and provides a novel basis for efforts to achieve compliance. Counselling and monitoring of dietary aspects in WD could be reinforced for effective management of patients.

Terminology

Fulminant hepatitis is the appearance of severe, life-threatening complications that occur rapidly after the first signs of liver disease (such as jaundice). The damage to the liver is broad, leading to the death of most (80%-90%) of the liver cells.

Peer review

This article is about the correlation between dietary copper level and the onset of fulminant hepatitis in the rat model of WD, especially at different time points. The article presents an interesting topic of WD to simulate the liver injury and onset of fulminant hepatitis. Although the model has been previously described the current study provides additional new value to the field.

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ORIGINAL ARTICLE

MUC5AC/β-catenin expression and *KRAS* gene alteration in laterally spreading colorectal tumors

Kosaburo Nakae, Hiroyuki Mitomi, Tsuyoshi Saito, Michiko Takahashi, Takashi Morimoto, Yasuhiro Hidaka, Naoto Sakamoto, Takashi Yao, Sumio Watanabe

Kosaburo Nakae, Takashi Morimoto, Yasuhiro Hidaka, Naoto Sakamoto, Sumio Watanabe, Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
Kosaburo Nakae, Hiroyuki Mitomi, Tsuyoshi Saito, Michiko Takahashi, Takashi Morimoto, Yasuhiro Hidaka, Takashi Yao, Department of Human Pathology, Juntendo University School of Medicine, 1-1-19 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
Author contributions: Nakae K, Mitomi H, Yao T and Watanabe S designed research; Nakae K, Takahashi M, Morimoto T and Hidaka Y performed research; Mitomi H, Saito T and Sakamoto N analyzed data; Nakae K and Mitomi H wrote the paper.
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Correspondence to: Hiroyuki Mitomi, MD, PhD, Department of Human Pathology, Juntendo University School of Medicine, Motomachi building 3F, 1-1-19 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. hmitomi@juntendo.ac.jp
Telephone: +81-3-58021037 Fax: +81-3-38121056
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Abstract

AIM: To clarify differences in mucin phenotype, proliferative activity and oncogenetic alteration among subtypes of colorectal laterally spreading tumor (LST).

METHODS: LSTs, defined as superficial elevated lesions greater than 10 mm in diameter with a low vertical axis, were macroscopically classified into two subtypes: (1) a granular type (Gr-LST) composed of superficially spreading aggregates of nodules forming a flat-based lesion with a granulonodular and uneven surface; and (2) a non-granular type (NGr-LST) with a flat smooth surface and an absence of granulonodular formation. A total of 69 LSTs, comprising 36 Gr-LSTs and 33 NGr-LSTs, were immunohistochemically stained with MUC2, MUC5AC, MUC6, CD10 (markers of gastrointestinal cell lineage), p53, β-catenin and Ki-67 antibodies, and examined for alteration in exon 1 of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) and exon 15 of v-raf murine sarcoma viral oncogene homologue B1 (BRAF) by polymerase chain reaction followed by direct sequencing.

RESULTS: Histologically, 15 Gr-LST samples were adenomas with low-grade dysplasia (LGD), 12 were high-grade dysplasia (HGD) and 9 were adenocarcinomas invading the submucosa (INV), while 12 NGr-LSTs demonstrated LGD, 14 HGD and 7 INV. In the proximal colon, MUC5AC expression was significantly higher in the Gr-type than the NGr-type. MUC6 was expressed only in NGr-LST. MUC2 or CD10 did not differ. P53 expression demonstrated a significant stepwise increment in progression through LGD-HGD-INV with both types of LST. Nuclear β-catenin expression was significantly higher in the NGr-type. Ki-67 expression was significantly higher in the Gr-type in the lower one third zone of the tumor. In proximal, but not distal colon tumors, the incidence of KRAS provided mutation was significantly higher in the Gr-type harboring a specific mutational pattern (G12V). BRAF mutations (V600E) were detected only in two Gr-LSTs.

CONCLUSION: The two subtypes of LST, especially in the proximal colon, have differing phenotypes of gastrointestinal cell lineage, proliferation and activation of Wnt/β-catenin or RAS/RAF/extracellular signal-regulated kinase signaling.

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Key words: Laterally spreading tumor; Mucin core protein; Colon; β-catenin; Immunohistochemistry; v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; Direct sequencing; Adenoma-carcinoma sequence

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INTRODUCTION

Colorectal cancer (CRC) is considered to arise from an adenoma precursor, and a model for genetic alterations in the adenoma-carcinoma sequence has been proposed [1,2]. In this model, an adenomatous polyposis coli (*APC*) gene mutation occurs at the earliest stage, followed by a v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation, as well as a change in p53. Along this sequence, the Wnt/APC/β-catenin and RAS/RAF/extracellular signal-regulated kinase (ERK) signaling pathways also play important roles[3-7]. Previous studies have primarily considered protruded adenomatous polyps as the most likely precursor of CRC[1,2]. However, recent advances have led to changes in the diagnosis of early colorectal tumors, which can now be morphologically divided into three groups: classical protruded tumors, depressed tumors, and laterally spreading tumors (LSTs)[8,9]. The latter two are possible candidates for alternative pathways of colorectal tumorigenesis. LSTs are considered to be less invasive, as neoplastic cells tend to spread along the surface of the lumen, and are usually categorized into two subtypes: granular type (Gr-LST) and flat- or non-granular type (NGr-LST); NGr-LSTs were more often associated with submucosal invasion compared to Gr-LSTs[9,10]. An earlier report demonstrated unique cell kinetics in LSTs[11]. Subsequent studies also evaluated alteration of APC[12] or β-catenin[12-15] for Wnt/APC/β-catenin, mutation of KRAS[11,12,14,16-19] or v-raf murine sarcoma viral oncogene homologue B1 (BRAF)[12,14,19] for RAS/RAF/ERK and mutation of phosphoinositide-3-kinase (PI3K) catalytic-α polypeptide[12,19] for the PI3K/AKT signaling pathway in LSTs. In addition, promoter methylation of CpG islands including its methylator phenotype (CIMP) in association with other genetic alterations has been shown to make important contributions to LST development[14,18].

In an earlier study, *de novo* appearance of gastric mucin genes MUC5AC and MUC6, which are present in surface foveolar cells and mucous neck cells of the oxyntic mucosa and antral-type glands in the stomach[20], was shown in colonic adenomas[21]. Altered expression of MUC2 as an intestinal apomucin known to be expressed in goblet cells[22], and CD10 expressed on the brush borders of intestinal epithelial cells[23], occurred during progression of adenoma to early adenocarcinoma state[24-26]. While considerable effort has been made to identify gene mutations and alteration of gastrointestinal phenotypes in conventional colorectal tumors, less attention has been paid to LSTs. Therefore, in the present study, we focused on differences in expression of MUC2, MUC5AC, MUC6 and CD10, p53 alteration, nuclear translocation of β-catenin, cell proliferation, and KRAS/BRAF mutations in morphologically different LST subtypes.

MATERIALS AND METHODS

Patients and materials

LSTs are defined as superficial elevated lesions greater than 10 mm in diameter with a low vertical axis[8-10] and are macroscopically classified into two subtypes: (1) Gr-LSTs composed of superficially spreading aggregates of nodules forming flat based lesions with a granulonodular and uneven surface (Figure 1A and C); and (2) NGr-LSTs with flat smooth surfaces and an absence of granulonodular formation (Figure 1B and D).

The material for our study was a series of 69 LSTs (from 69 patients) resected endoscopically or surgically at Jundendo University Hospital (Tokyo, Japan) between May 2008 and July 2011. Patients with familial polyposis coli, hereditary non-polyposis colorectal carcinoma, multiple colorectal carcinomas, or inflammatory bowel disease were not included in the analysis. Proximal LSTs were classified as tumors proximal to the splenic flexure and the remaining tumors were defined as distal. Resected specimens were fixed in 15% formalin and processed for embedding in paraffin wax according to routine procedures and then sections were cut and stained with hematoxylin and eosin (H and E). This study was approved by the Institutional Review Board and the ethical committee of our hospital.

Pathological review

All slides stained with H and E were reviewed by two experienced gastrointestinal pathologists (Mitomi H, Yao T) independently. According to the World Health Organization classification[27], adenoma was classified as tubular, tubulovillous or villous type with low grade dysplasia (LGD) or high grade dysplasia (HGD). Intramucosal carcinoma and carcinoma *in situ* were included in adenoma with HGD. Adenocarcinoma invading submucosa (INV) was classified as well, moderately or poorly differentiated. The subjects were classified according to the most advanced lesion identified. Interobserver variation was resolved by reevaluation and discussion to reach consensus.

Immunohistochemistry

Briefly, 4 μm thick tissue serial sections were dewaxed in xylene and rehydrated, then endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxidase. For heat-induced antigen retrieval, sections were transferred to citrate buffer (10 mmol/L, pH 6.0) and heated at 100 ℃ in a water bath. The primary antibodies employed were MUC2 (Ccp58, 1:100 dilution; Leica Biosystems/Novocastra Laboratories, Newcastle Upon Tyne, UK), MUC5AC (CLH2, 1:100 dilution; Novocastra Laboratories), MUC6 (CLH5, 1:100 dilution; Novocastra Laboratories), CD10 (56C6, 1:100 dilution; Novocastra Laboratories), p53 (PAb 1801, 1:100; Leica Biosystems/Novocastra Laboratories), β-catenin (14/Beta-Catenin,

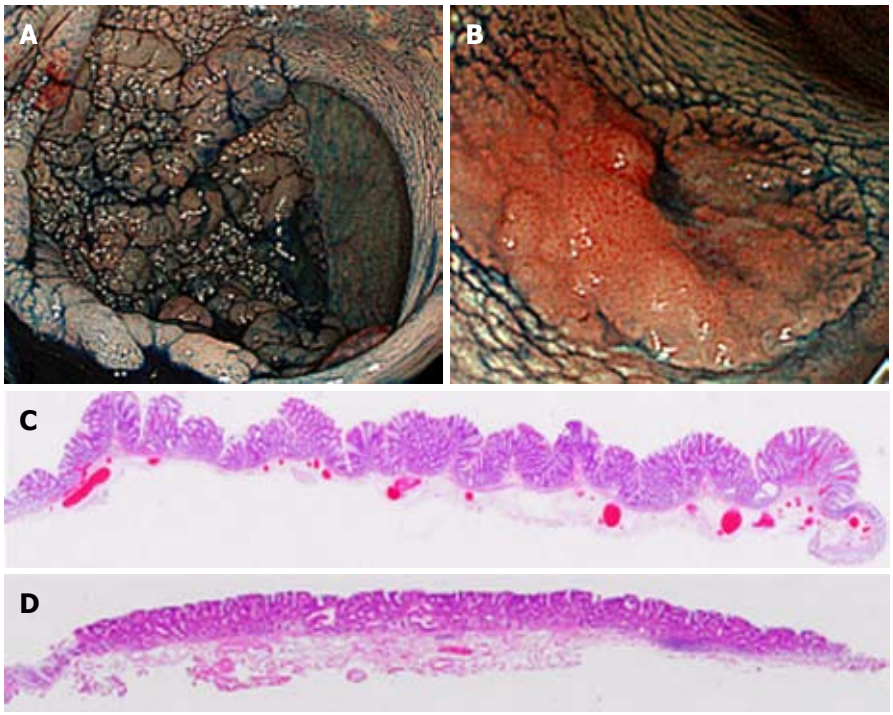


Figure 1 Macroscopic appearance (chromoscopic image) of laterally spreading tumors. (A) Granular type (Gr-LST) and (B) flat- or non-granular type (NGr-LST). Whole mount view of cut sections of LSTs stained with hematoxylin and eosin: (C) A Gr-LST showing tubulovillous structures with nodular surfaces and (D) an NGr-LST consisting of small tubular glands with flat surfaces. LSTs: Laterally spreading tumors.

dilution 1:100, BD Biosciences, San Diego, CA), and Ki-67 (MIB-1, 1:100; DAKO, Glostrup, Denmark), with incubation for 120 min at room temperature. Immunostaining was performed using the streptavidin-biotin-peroxidase complex method using a Histofine SAB-PO Kit (Nichirei Corp., Tokyo, Japan). The chromogen was 3,3' -diaminobenzidine and the sections were counterstained lightly with Mayer's hematoxylin to facilitate recognition of structures.

Assessment of immunostaining

Distinct cytoplasmic staining for MUC2, MUC5AC, MUC6 and apical staining for CD10 were considered positive, as was nuclear staining for p53 and Ki-67, regardless of the staining intensity. Expression of β-catenin, which generally showed an inverse relationship between membranous and nuclear (with cytoplasmic) reactivity, was evaluated only with respect to nuclear localization in this study. Immunostaining for all markers was evaluated by two of the authors (Mitomi H, Nakae K) independently, without prior knowledge of clinicopathological data. Discrepancies were resolved by re-evaluation to reach consensus.

Immunoreactive scores (IRSs) for MUC2, MUC5AC, MUC6 and CD10 were classified into five grades: 0 points, positive cells < 5% of tumor area; 1 point, 5%-24%; 2 points, 25%-49%; 3 points, 50%-74%; 4 points, ≥ 75%. IRSs for p53 and nuclear β-catenin were also classified into five grades: 0 points, positive nuclei < 5% of tumor cells; 1 point, 5%-24%; 2 points, 25%-49%; 3 points, 50%-74%; 4 points, ≥ 75%. For topological evaluation of the Ki-67 labeling index (LI; %), tumor glands in the lamina propria were separated into three equal zones (upper, middle and lower thirds), and the number of immunoreactive nuclei per approximately 300 tumor cells were counted in each zone (for a total of approximately 1000 cells in whole glands).

Mutation analysis of KRAS and BRAF

Mutation analysis for KRAS and BRAF were performed using genomic DNA derived from formalin-fixed paraffin-embedded tissue of 29 Gr-LSTs (10 LGD, 10 HGD and 9 INV) and 27 NGr-LSTs (10 LGD, 10 HGD and 7 INV). Mutations were examined in exon 1 of KRAS and exon 15 of BRAF by polymerase chain reaction followed by direct sequencing. The primer sequences in this study were as previously described[28].

Statistical analysis

All statistical analysis were carried out using StatView for Windows Version 5.0 (SAS Institute Inc., Cary, NC, United States). IRSs and LI are presented as mean ± SD. Continuous data were compared with the Mann-Whitney *U* test. Categorical analysis of variables was performed using either the χ^2 test (with Yates' correction) or the Fisher's exact test, as appropriate. Correlations among expression levels of the encoded proteins were assessed with the Spearman's rank correlation coefficient. A P value < 0.05 was considered statistically significant, with classification into two grades: $P < 0.05$ and $P < 0.01$.

RESULTS

Clinicopathological characteristics of LST

The clinicopathological characteristics of the Gr-LSTs (15 LGD, 12 HGD, 9 INV) and NGr-LSTs (12 LGD, 14 HGD, 7 INV) are shown in Table 1. Gr-LSTs were located equally in proximal and distal colons, whereas NGr-LSTs were more frequently found in the distal colon, but without reaching statistical significance. Gr-LSTs were

Table 1 Clinicopathological characteristics of laterally spreading tumor of the colorectum

Variable	Gr-type (n = 36)	NGr-type (n = 33)
Age (yr)	68.5 ± 10.9 (34-88)	69.0 ± 10.3 (44-85)
Sex		
Male	21	23
Female	15	10
Location		
Proximal colon	18	12
Distal colon	18	21
Size of tumor (mm)	35.3 ± 17.9 (12-80)[b]	23.0 ± 9.1 (10-50)[b]
Histology		
LGD	15	12
Tubular type	11	11
Tubulovillous type	4	1
HGD	12	14
Tubular type[d]	0	13
Tubulovillous type[d]	12	1
INV	9	7
Well differentiated type	7	7
Others	2	0

Age and size of tumor are represented as mean ± SD (range); Gr-type: Granular type of laterally spreading tumor; NGr-type: Flat- or non-granular type of laterally spreading tumor; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading submucosa; INV-others: Mixed well and moderately differentiated type (n = 1) and mixed well and poorly differentiated adenocarcinoma (n = 1); [b,d]$P < 0.01$, Gr-type *vs* NGr-type, tubular type *vs* tubulovillous type.

significantly larger than NGr-LSTs. Histologically, 12 out of 13 (92%) tubulovillous adenomas with HGD were Gr-LSTs, whereas all of the 13 tubular adenomas with HGD were NGr-LSTs. All NGr-INVs and 7 out of 9 (78%) Gr-INVs were well differentiated adenocarcinomas. The two other Gr-INVs were an adenocarcinoma coexisting with well and moderately differentiated histology, and a mixed well and poorly differentiated type adenocarcinoma.

IRSs of MUC2, MUC5AC, MUC6, CD10, p53 and nuclear β-catenin in LSTs

For the MUC2 IRS (Figure 2A-C), significant stepwise decrement was evident in progression through LGD - HGD-INV in Gr- and NGr-LSTs. For the MUC5AC IRS (Figure 2D-F), the Gr-type value was significantly higher than that of the NGr-type, in the proximal colon. MUC6 was expressed only in NGr-LSTs (Figure 2G-I). A low extent of CD10 expression was detected in HGD and INV, but not LGD of both Gr- and NGr-LSTs (Figure 2J-L).

For the p53 IRS (Figure 3A), a stepwise increment in progression was noted through LGD to INV in Gr- and NGr-LSTs, which was statistically significant. For the nuclear β-catenin IRS (Figure 3B), the NGr-type value was significantly higher than the Gr-type. A similar trend for p53 and nuclear β-catenin IRSs was observed in proximal and distal LSTs (data not shown). For the Ki-67 LI (%) in the lower zone (Figure 3C), the Gr-type value was significantly higher than that of the NGr-type. A similar trend without statistical significance was found in the upper and middle zones (data not shown).

Representative H and E features and expression of MUC2, MUC5AC, MUC6, CD10, p53 and β-catenin are illustrated in Figure 4.

Correlations among IRSs for the encoded proteins

Data for correlations among IRS are given in Table 2. Significant inverse relationships were found between p53 and MUC2 or MUC5AC. In addition, inverse associations were shown between nuclear β-catenin and MUC2 in NGr-LSTs, or MUC5AC in Gr-LSTs. Reciprocally, inverse associations were noted between Ki-67 and MUC2 in Gr-LSTs, or MUC5AC in NGr-LSTs.

KRAS and BRAF mutations

The results for KRAS and BRAF mutations analyzed are shown in Table 3. In proximal colon lesions, the incidence of KRAS mutations was significantly higher in Gr-LSTs (69%) than in NGr-LSTs (6%; $P < 0.001$). BRAF mutations were only detected in distal Gr-LSTs. In Gr-LSTs, 7 out of 29 (24%) tumors showed mutations from wild-type GGC (glycine) to GAC (aspartic acid) in codon 13 of the *KRAS* gene, and 5 (17%) tumors harbored mutations from GGT (glycine) to GTT (valine) in codon 12. In NGr-LSTs, 2 out of 27 (7%) tumors had mutations from GGC (glycine) to GAC (aspartic acid) in codon 13 of the *KRAS* gene. BRAF mutations were GTG (valine) to GAG (glutamic acid) in codon 600. A schematic representation of KRAS and BRAF mutational patterns is shown in Figure 5.

Association of KRAS or BRAF mutations with clinicopathological parameters and IRSs of the encoded proteins

Since there were only two cases of NGr-LST harbored mutations of KRAS, and neither had BRAF mutations, we analyzed only Gr-LSTs with respect to the relationship of KRAS or BRAF mutations with clinicopathological parameters (age, sex, location, size of tumor, and dysplastic grade) and IRSs of the encoded proteins. No significant link was observed with clinicopathological parameters. The MUC5AC IRS was significantly higher in mutated Gr-LSTs compared to non-mutated tumors (1.2 ± 1.3 *vs* 0.2 ± 0.6, $P < 0.05$). No significant differences were found in other IRSs (data not shown).

DISCUSSION

Only a few reports are available on the phenotypic expression of gastrointestinal cell lineage with immunohistochemistry of MUC2, MUC5AC, MUC6 and CD10 in relation to colorectal adenoma-carcinoma sequence[24-26]. In proximal colon lesions, MUC5AC expression was significantly higher in Gr-LSTs than NGr-LSTs. Expression of MUC5AC or HGM was increased in adenoma with villous histology or polypoid growth[21,24,25]. Enhanced MUC6 immunoreactivity was reported to be exhibited in large adenomas[21]. In the current study, MUC6 was expressed only in NGr-LSTs, suggesting it as a marker of a morphologically distinct tumor. The molecular mechanisms responsible for the aberrant expression of gastric mucin,

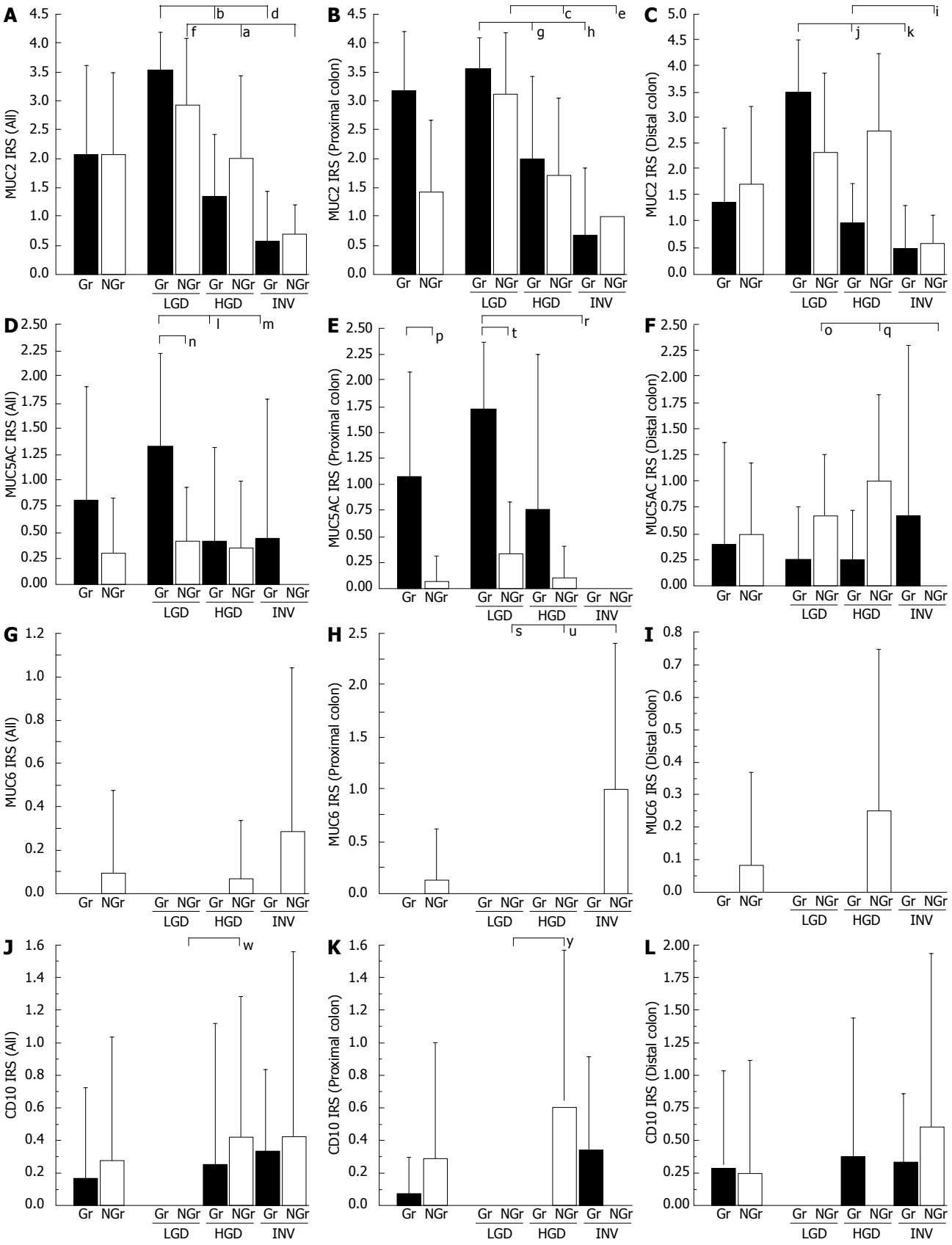


Figure 2 Immunoreactive scores for MUC2, MUC5AC, MUC6 and CD10 in all (A, D, G, J), proximal (B, E, H, K) and distal (C, F, I, L) laterally spreading tumors. Gr, Gr-LST (black bar); NGr, NGr-LST (white bar). IRS: Immunoreactive score; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading into submucosa; LSTs: laterally spreading tumors; Gr: Granular; NGr: Non-granular. Data are mean ± SD; $^{a, c, e, g, i, k, m, o, q, s, u, w, y}P < 0.05$, MUC2: all NGr-HGD *vs* NGr-INV; proximal colon NGr-LGD *vs* NGr-HGD or NGr-INV; Gr-LGD *vs* Gr-HGD; distal colon Gr-LGD *vs* Gr-INV; NGr-HGD *vs* NGr-INV; MUC5AC: all Gr-LGD *vs* Gr-INV; distal colon NGr-INV *vs* NGr-HGD or NGr-LGD; MUC6: proximal colon NGr-INV *vs* NGr-LGD or NGr-HGD; CD10: all NGr-LGD *vs* NGr-HGD; proximal colon NGr-LGD *vs* NGr-HGD; $^{b, d, f, h, j, l, n, p, r, t}P < 0.01$, MUC2: all Gr-LGD *vs* Gr-HGD or Gr-INV; NGr-LGD *vs* NGr-INV; proximal colon Gr-LGD *vs* Gr-INV; distal colon Gr-LGD *vs* Gr-HGD; MUC5AC: all Gr-LGD *vs* NGr-LGD; Gr-LGD *vs* Gr-HGD; proximal colon Gr-type *vs* NGr-type; Gr-LGD *vs* NGr-LGD; Gr-LGD *vs* Gr-INV.

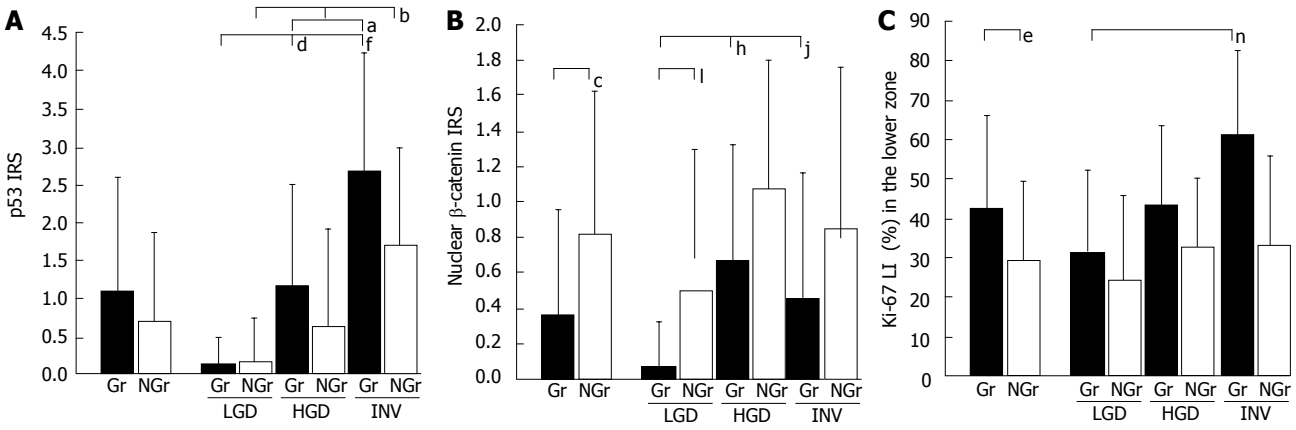


Figure 3 Immunoreactive scores for p53 (A) and nuclear β-catenin (B), and labeling indices for Ki-67 in the lower zone (C) in all laterally spreading tumors. Gr, Gr-LST (black bar); NGr, NGr-LST (white bar); LSTs: laterally spreading tumors. Data are mean ± SD; [a, c, e]$P < 0.05$, p53: Gr-HGD *vs* Gr-INV; nuclear β-catenin: Gr-type *vs* NGr-type; Ki-67, Gr-type *vs* NGr-type; [b, d, f, h, j, l, n]$P < 0.01$, p53: NGr-LGD *vs* NGr-INV; Gr-LGD *vs* Gr-HGD or Gr-INV; nuclear β-catenin: Gr-LGD *vs* Gr-HGD or Gr-INV: Gr-LGD *vs* NGr-LGD; Ki-67, Gr-LGD *vs* NGr-INV. IRSs: Immunoreactive scores; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading into submucosa; Gr: Granular; NGr: Non-granular; LI: Labeling indice.

Table 2 Correlations among expression levels of the encoded proteins

	MUC5AC		MUC6		CD10		p53		Nuclear β-catenin		Ki-67
	Gr-type	NGr-type	Gr-type	NGr-type	Gr-type	NGr-type	Gr-type	NGr-type	Gr-type	NGr-type	Gr-type
MUC2	0.550[b]	0.486[b]	NS	NS	-0.417[a]	NS	-0.549[a]	-0.450[a]	NS	0.413[a]	-0.397[a]
MUC5AC	Blank		NS	NS	NS	NS	-0.430[a]	-0.368[a]	-0.334[a]	NS	NS
MUC6	Blank		Blank		NS	NS	NS	NS	NS	NS	NS
CD10	Blank		Blank		Blank		NS	NS	NS	NS	NS
p53	Blank		Blank		Blank		Blank		NS	NS	0.514[b]
Nuclear β-catenin	Blank		Blank		Blank		Blank		Blank		NS

Gr-type: Granular type of laterally spreading tumor; NGr-type: Flat- or non-granular type of laterally spreading tumor; Data indicates rho value examined by Spearman's correlation coefficient; NS: Not significant. [a]$P < 0.05$; [b]$P < 0.01$.

Table 3 Frequency of KRAS and BRAF mutations in laterally spreading tumor of the colorectum

Type of tumors	Number (%) of mutated samples				
	All			Proximal colon	
	n	KRAS	BRAF	*n*	KRAS
Gr-type	29	15 (52)[a]	2 (7)	13	9 (69)[c]
LGD	10	6 (60)	0 (0)	7	6 (86)[d,e]
HGD	10	6 (60)[b]	1 (10)	3	2 (67)
INV	9	3 (33)	1 (11)	3	1 (33)
NGr-type	27	2 (7)[a]	0 (0)	18	1 (6)[c]
LGD	10	1 (10)	0 (0)	8	1 (13)[d]
HGD	10	0 (0)[b]	0 (0)	8	0 (0)
INV	7	1 (14)	0 (0)	2	0 (0)
Total	56	17 (30)	2 (4)	30	10 (33)

Gr-type: Granular type of laterally spreading tumor; NGr-type: Flat- or non-granular type of laterally spreading tumor; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading submucosa; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; BRAF: v-raf murine sarcoma viral oncogene homologue B1. [a, c, e]$P < 0.05$, All and proximal colon: Gr-type *vs* NGr-type; Gr-LGD: proximal *vs* distal colon; [b, d]$P < 0.01$, all and proximal colon: Gr-HGD *vs* NGr-HGD; proximal colon: Gr-type *vs* NGr-type.

MUC5AC and MUC6 in colorectal tumors are still unclear and may be due to changes in transcriptional regulation.

It was previously reported that none of the four NGr-LSTs harbored p53 mutations, whereas 7 out of 24 (29%) Gr-LSTs were positive[16]. We have shown that no significant differences in p53 expression, along with an inverse relation of p53 to MUC2 or MUC5AC expression in two types of LSTs. An experimental study identified MUC2 expression as increased along with induction of wild-type p53 in carcinoma cell lines *in vitro*, and potential p53-binding sites in the MUC2 promoter, which contributes to stimulation of promoter activity[29]. P53 overexpression as an indirect sign of loss of functional p53 by its mutation is possibly related to the down-regulation of MUC2 expression in colorectal LST. A detailed study on the relation of p53 to the altered mucin expression appears warranted.

We have documented high nuclear β-catenin expression in NGr-LSTs in line with a previous study[15]. In an analysis of LSTs, no mutation of β-catenin exon 3 was found, while LOH at 5q (APC locus) was more frequently detected in NGr-LSTs than in Gr-LSTs[12]. To date, there is only one case report of NGr-LST harboring interstitial deletion of β-catenin exon 3[13]. Nuclear accumulation of β-catenin has been observed in tumors with mutations in the β-catenin or APC gene[3]. Taken together, the available data suggest that activation of Wnt/APC/β-catenin signaling in NGr-LSTs is due primarily to alterations in the *APC* gene. Furthermore, in-

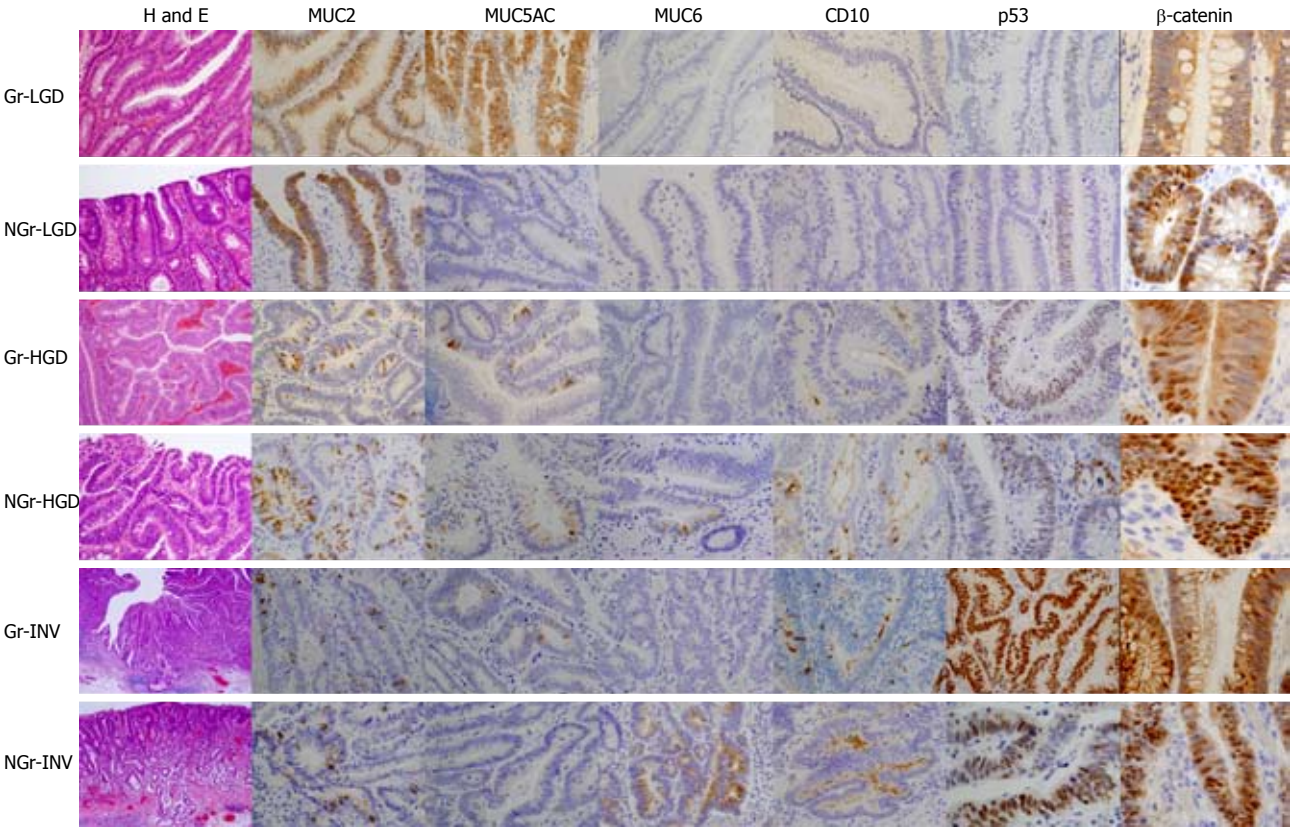


Figure 4 Histology and immunohistochemistry of laterally spreading tumors. Immunoreactive scores (IRSs) for Gr-LGD: MUC2, 4 points; MUC5AC, 2 points; MUC6, 0 points; CD10, 0 points; p53, 0 points; nuclear β-catenin, 0 points. IRSs for NGr-LGD: MUC2, 3 points; MUC5AC, 1 points; MUC6, 0 points, CD10, 0 points; p53, 0 points; nuclear β-catenin, 1 points. IRSs for Gr-HGD: MUC2, 1 points; MUC5AC, 1 points; MUC6, 0 points, CD10, 1 points; p53, 1 points; nuclear β-catenin, 1 points. IRSs for NGr-HGD: MUC2, 2 points; MUC5AC, 1 points; MUC6, 1 points, CD10, 1 points; p53, 1 points; nuclear β-catenin, 2 points. IRSs for Gr-INV: MUC2, 1 points; MUC5AC, 1 points; MUC6, 0 points, CD10, 1 points; p53, 4 points; nuclear β-catenin, 1 points. IRSs for NGr-INV: MUC2, 1 points; MUC5AC, 0 points; MUC6, 2 points, CD10, 2 points; p53, 2 points; nuclear β-catenin, 2 points (H and E, ×80; immunoperoxidase: MUC2, MUC5AC and MUC6, ×100; CD10 and p53, ×120; β-catenin, ×300); LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading into submucosa; Gr: Granular; NGr: Non-granular.

verse associations were shown between nuclear β-catenin and MUC2 or MUC5AC in LSTs. Experimental studies have demonstrated that abrogation of MUC2 in tumors of the rat colon is related to nuclear β-catenin localization and its mutation[30,31]. Interaction of mucin core protein with Wnt/APC/β-catenin signaling may have some role in the progression of LSTs.

Ki-67, considered to be a reliable indicator for accurately assessing growth fraction, is increased with a shift in the proliferative zone toward the upper compartment in LSTs[11]. In the current study of Ki-67, a similar upward shift was detected in Gr- and NGr-LSTs. Furthermore, higher proliferation in the lower compartment was more apparent in Gr-LSTs, which may explain the morphologic variation in LSTs. We also found an inverse association between tumor cell proliferation (Ki-67) and MUC2 in LST. This is in line with the fact that decreased *in vivo* expression of MUC2 is related to colon carcinogenesis, accompanied by increased cell proliferation[32].

We have shown that, in proximal colon, the incidence of KRAS mutation was significantly higher in Gr-LST (69%) than NGr-LST (6%), with a relatively frequent and specific pattern in Gr-type for G12V, as it was for G12C in another report[16]. Previously reported incidences of KRAS mutation were 21%-83% in Gr-LST and 17%-26% in NGr-LST[12,16,17-19]. In the current study, BRAF mutations (V600E) were only detected in two Gr-LSTs. Gr-LSTs, particularly those located in the proximal colon, exhibited frequent KRAS mutations and high CIMP[18]. BRAF mutations are often characteristic of CIMP-high/microsatellite instability-high colorectal cancer[6], and are infrequent in LST[12,14,19]. These facts suggest that proximal Gr-LST is a possible candidate for early lesions of CIMP-high/microsatellite stable cancer. Furthermore, MUC5AC expression was significantly higher in KRAS mutated Gr-LSTs than in non-mutated tumors. Aberrant MUC5AC expression is thought to be related to KRAS mutations in experimental colon carcinogenesis[31]. *In vitro*, upregulation of MUC5AC may occur through concomitant activation of the EGFR/RAS/RAF/ERK signaling pathway and Sp1 binding to the gene promoter[33]. We therefore hypothesize that ERK signal activation induced by mutated RAS in relation to aberrant gastric mucin expression may play in a role in the development and progression of Gr-LSTs in the proximal colon.

In conclusion, as summarized schematically in Figure 6, the two subtypes of LST have differing mucin pheno-

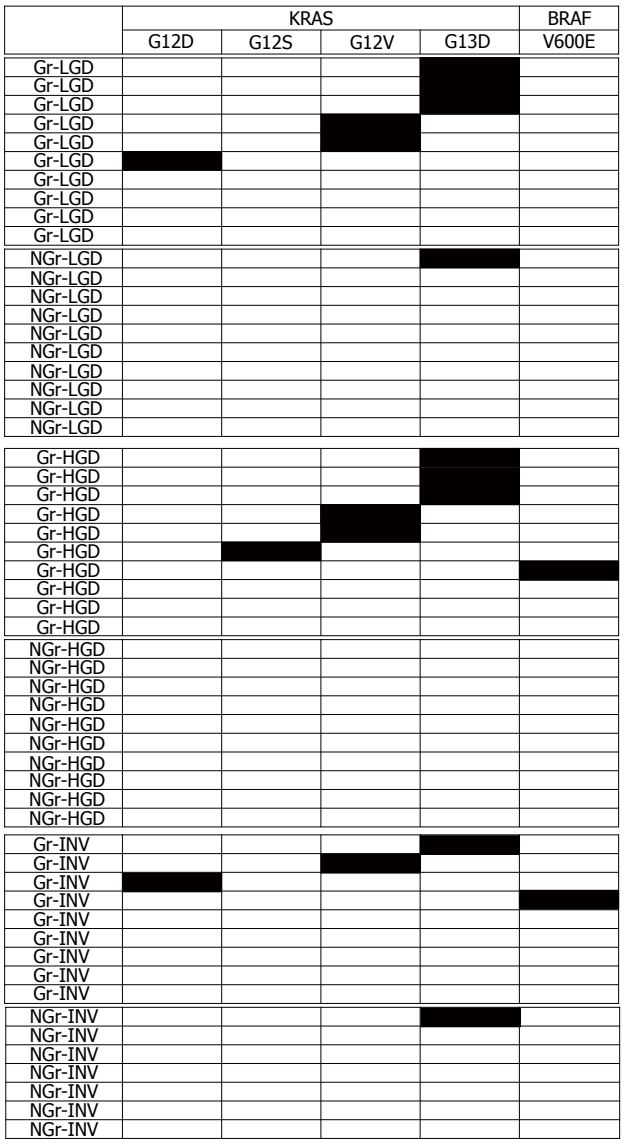


Figure 5 v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog and v-raf murine sarcoma viral oncogene homologue B1 mutational patterns in laterally spreading tumors. D: Aspartic acid; E: Glutamic acid; G: Glycine; S: Serine; V: Valine; Black box: Mutated case; White box: Non-mutated case; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading into submucosa; Gr: Granular; NGr: Non-granular; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; BRAF: v-raf murine sarcoma viral oncogene homologue B1.

typic expression, proliferative activity, and activation of Wnt/β-catenin or RAS/RAF/ERK signaling in progression from adenomas through to invasive carcinomas.

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COMMENTS

Background

Laterally spreading tumors (LSTs) in the colorectum are usually categorized into two subtypes: granular (Gr-LST) and flat- or non-granular types (NGr-LST). While considerable effort has been made to identify gene mutations and alteration of gastrointestinal phenotypes in conventional colorectal tumors, less attention has been paid to LSTs.

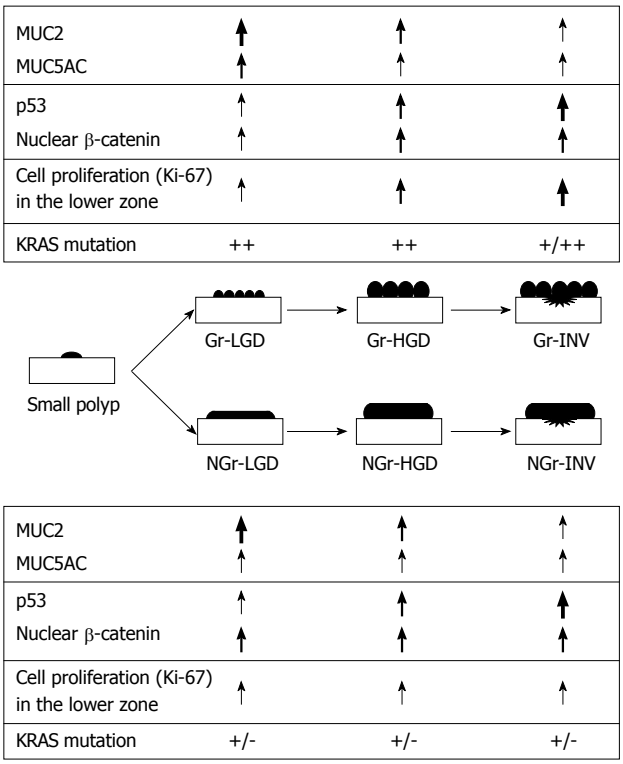


Figure 6 Alterations of expression of mucin core protein, p53 and β-catenin, cell proliferation and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog mutations in malignant transformation of laterally spreading tumors. Large arrow: Marked upregulation; Medium arrow: Moderate upregulation; Small arrow: Mild upregulation; ++: Frequently mutated; +: Infrequently mutated; -: Not mutated; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading into submucosa; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; Gr: Granular; NGr: Non-granular.

Research frontiers

The authors focused on differences in expression of MUC2, MUC5AC, MUC6 and CD10, p53 alteration, nuclear translocation of β-catenin, cell proliferation, and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)/v-raf murine sarcoma viral oncogene homologue B1 mutations in morphologically different LST subtypes.

Innovations and breakthroughs

The authors showed that the two types of LSTs have different phenotypes, particularly with respect to MUC5AC (expression greater in Gr- *vs* NGr- types) and MUC6 (only expressed in NGr-type). They showed a higher nuclear β-catenin expression in NGr-type, and Ki-67 was much more prevalent in the Gr-type. Finally, the incidence of KRAS mutations was much more frequent in Gr-LST.

Applications

The subtypes of LSTs may be different candidates for alternative pathways of colorectal tumorigenesis. The results of the study represent a further impact on research in colorectal carcinogenesis.

Peer review

This is a good descriptive study in which the authors clarify differences in mucin phenotype, proliferative activity and oncogenetic alteration among subtypes of LST. The results are interesting and suggest that they are different candidates for alternative pathways of colorectal carcinogenesis.

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BRIEF ARTICLE

Software for automated classification of probe-based confocal laser endomicroscopy videos of colorectal polyps

Barbara André, Tom Vercauteren, Anna M Buchner, Murli Krishna, Nicholas Ayache, Michael B Wallace

Barbara André, Tom Vercauteren, Image Computing Group, Mauna Kea Technologies, 75010 Paris, France
Barbara André, Nicholas Ayache, Asclepios Research Team, The National Institute for Research in Computer Science and Control Sophia Antipolis, 06902 Sophia Antipolis, France
Anna M Buchner, Division of Gastroenterology, Hospital of the University of Pennsylvania, Philadelphia, PA 19104, United States
Murli Krishna, Laboratory Medicine and Pathology, Mayo Clinic Hospital in Jacksonville, Jacksonville, FL 3224, United States
Michael B Wallace, Department of Gastroenterology and Hepatology, Mayo Clinic Hospital in Jacksonville, Jacksonville, FL 3224, United States
Author contributions: André B was involved in study concept and design, analysis and interpretation of data, drafting of the manuscript and statistical analysis; Vercauteren T was involved in study concept and design, analysis and interpretation of data and study supervision; Buchner AM was involved in acquisition of data and analysis and interpretation of data; Krishna M was involved in analysis and interpretation of data; Ayache N was involved in study concept and design, analysis and interpretation of data and study supervision; Wallace MB was involved in acquisition of data, analysis and interpretation of data and study supervision.
Correspondence to: Barbara André, PhD, Image Computing Group, Mauna Kea Technologies, 9 rue d'Enghien, 75010 Paris, France. barbara.andre@maunakeatech.com
Telephone: +33-1-70080961 Fax: +33-1-48241218
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Abstract

AIM: To support probe-based confocal laser endomicroscopy (pCLE) diagnosis by designing software for the automated classification of colonic polyps.

METHODS: Intravenous fluorescein pCLE imaging of colorectal lesions was performed on patients undergoing screening and surveillance colonoscopies, followed by polypectomies. All resected specimens were reviewed by a reference gastrointestinal pathologist blinded to pCLE information. Histopathology was used as the criterion standard for the differentiation between neoplastic and non-neoplastic lesions. The pCLE video sequences, recorded for each polyp, were analyzed off-line by 2 expert endoscopists who were blinded to the endoscopic characteristics and histopathology. These pCLE videos, along with their histopathology diagnosis, were used to train the automated classification software which is a content-based image retrieval technique followed by *k*-nearest neighbor classification. The performance of the off-line diagnosis of pCLE videos established by the 2 expert endoscopists was compared with that of automated pCLE software classification. All evaluations were performed using leave-one-patient-out cross-validation to avoid bias.

RESULTS: Colorectal lesions (135) were imaged in 71 patients. Based on histopathology, 93 of these 135 lesions were neoplastic and 42 were non-neoplastic. The study found no statistical significance for the difference between the performance of automated pCLE software classification (accuracy 89.6%, sensitivity 92.5%, specificity 83.3%, using leave-one-patient-out cross-validation) and the performance of the off-line diagnosis of pCLE videos established by the 2 expert endoscopists (accuracy 89.6%, sensitivity 91.4%, specificity 85.7%). There was very low power (< 6%) to detect the observed differences. The 95% confidence intervals for equivalence testing were: -0.073 to 0.073 for accuracy, -0.068 to 0.089 for sensitivity and -0.18 to 0.13 for specificity. The classification software proposed in this study is not a "black box" but an informative tool based on the query by example model that produces, as intermediate results, visually similar annotated videos that are directly interpretable by the endoscopist.

CONCLUSION: The proposed software for automated classification of pCLE videos of colonic polyps achieves high performance, comparable to that of off-line diagnosis of pCLE videos established by expert endoscopists.

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Key words: Colorectal neoplasia; Computer-aided diagnosis; Content-based image retrieval; Nearest neighbor classification software; Probe-based confocal laser endomicroscopy

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INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related death in the United States[1]. Its development includes several morphological stages, from benign to adenomatous polyps with low grade dysplasia to adenocarcinoma. Suspicious lesions are usually detected with standard colonoscopy by endoscopists who either perform confirmatory biopsy, or if high certainty exists, perform immediate therapy such as resection or ablation of diseased tissue. Because standard endoscopic imaging can only diagnose disease states with moderate levels of certainty[2,3], histopathology remains the criterion standard for final diagnosis[4]. However, the requirement for *ex vivo* histology implies a large proportion of unnecessary polypectomies and often requires a separate endoscopic procedure to be performed for treatment. It also increases the cost of colorectal cancer screening.

Probe-based confocal laser endomicroscopy (pCLE, Mauna Kea Technologies, France) enables the endoscopist to image the epithelial tissue *in vivo*, at the microscopic level with a confocal miniprobe, and in real-time during ongoing endoscopy. Preliminary findings by Meining *et al*[5] demonstrated the applicability of pCLE in diagnosing colorectal neoplasia *in vivo* with high sensitivity and specificity (93% and 92%, respectively) in 13 patients with colorectal lesions. Venkatesh *et al*[6] and De Palma[7] pointed out that confocal endomicroscopy offers the ability to target biopsies much more precisely and thus to reduce the number of random biopsies. In a recent study including a large pool of 75 patients, Buchner *et al*[8] compared off-line diagnosis of pCLE videos to virtual chromoendoscopy (Narrow-Band Imaging and Fujinon Intelligent Color Enhancement) and showed that off-line diagnosis of pCLE videos had higher sensitivity (91% *vs* 77%) with similar specificity (76%). As noted by Wallace *et al*[9], endoscopists now have the challenging task of performing "optical biopsies" and diagnosing pCLE video sequences *in vivo*.

In order to provide an objective support for pCLE diagnosis, we aimed to design a computer-based system for the automated classification of colonic polyps into neoplastic and non-neoplastic lesions. As physicians typically rely on similarity-based reasoning to establish a diagnosis from image queries, we propose a content-based image retrieval (CBIR) approach to automatically estimate the pathology of a new pCLE video. Indeed, contrary to "black box" classification systems, a CBIR-based classification system extracts, from a training database, annotated pCLE videos that are visually similar to the video of interest and directly interpretable by the endoscopist. The pathology of the video query is then estimated from the histopathological votes of these already diagnosed videos. Another advantage of CBIR-based classification is that the extracted similar videos can be presented to the endoscopist in a second reader paradigm to better support pCLE diagnosis.

The main goal of this study was to compare, using the same database of colonic polyps, the clinical performance of our automated pCLE classification software with that of off-line diagnosis of pCLE videos established by endoscopists expert in pCLE, with histopathology remaining the criterion standard reference.

MATERIALS AND METHODS

Patients

The patients included in the study were enrolled between November 2007 and March 2009 for previous studies approved by the Mayo Clinic Institutional Review Board, and from which we collected all available data to ensure as large a sample size as possible. These patients were enrolled into the study of Buchner *et al*[8] and for further studies by the same Mayo Clinic group. Only the patients with complete diagnostic data were considered in our study. All study participants gave full written consent. Patients were enrolled if they were due for surveillance or screening colonoscopies, evaluation of known or suspected polyps on other imaging modalities, and endoscopic mucosal resection of larger flat colorectal neoplasia. Exclusion criteria were patients with non-corrected coagulopathy, women who were pregnant or breast feeding, those with documented allergy to fluorescein, and patients with no colorectal lesions found during a study colonoscopy. Twenty-four hours before the procedure, patients were prepped with 2-4 L polyethylene glycol solution. Conscious sedation was performed with intravenous administration of midazolam and meperidine.

Endoscopy equipment and procedure

All procedures were performed by the authors (either Wallace MB or Buchner AM) using a high-definition colonoscope (Fujinon EC450HL5 or 490 ZW, Fujinon, Ft Wayne, NJ, United States; Olympus CFH180, Olympus, Center Valley, NY, United States). The system was equipped with the EPX 4400 processor (Fujinon Inc.) or CV 180 Exera (Olympus, Co.). The primary screening method was white-light high-definition colonoscopy. Then, either Fujinon Intelligent Color Enhancement

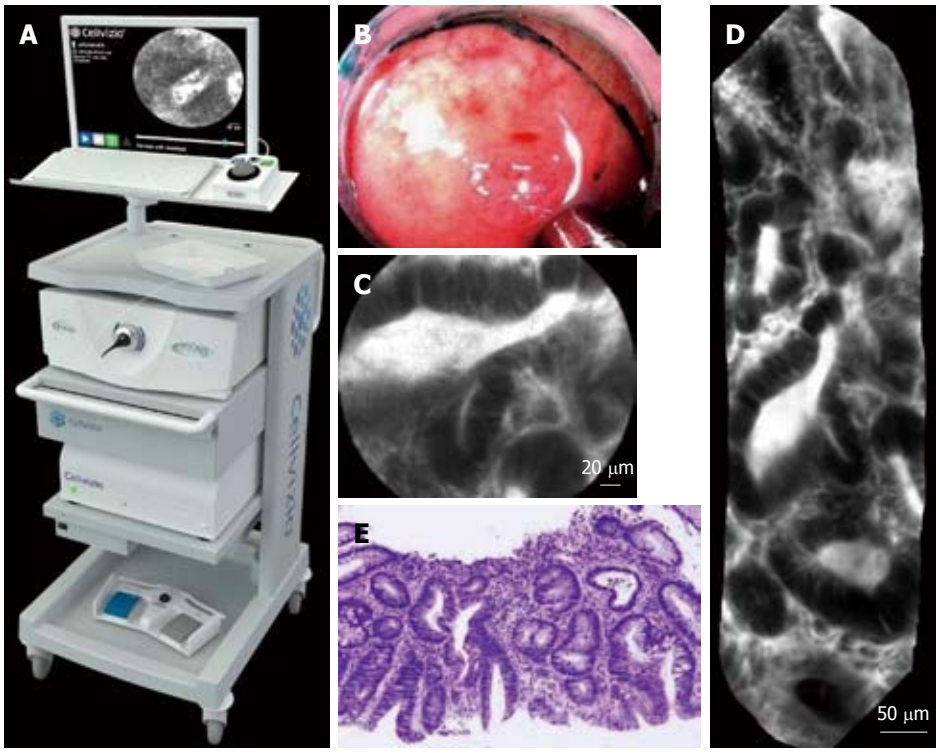


Figure 1 Imaging modalities of colonic polyps. A: Setup of probe-based confocal laser endomicroscopy (pCLE) imaging system (Cellvizio, Mauna Kea Technologies); B: Endoscopic image of tubular adenoma, and the pCLE miniprobe; C: An image of the pCLE video sequence; D: A pCLE mosaic image built with the video mosaicing tool; E: Histopathology image.

mode 4 with a Fujinon colonoscope or Narrow-Band Imaging with an Olympus 180 series scope was used to characterize lesions in all patients.

The surface pit pattern of the lesion was classified according to Kudo criteria. Anatomical site and morphological class of lesions were recorded in accordance with the Paris classification[10]. Fluorescein sodium 2.5-5.0 mL 10% (AK Fluor, Akorn Pharmaceutical, Lake Forest, IL, United States) solution was administered intravenously after the first polyp was identified. Immediately after fluorescein injection, pCLE video sequences of the lesions were acquired and recorded. According to the visual examination of both endoscopic and pCLE images, real biopsies were targeted to the most suspicious parts of the polyp. Appropriate treatment procedures, ranging from simple polypectomies to complex endoscopic mucosal resection of lesions, were then performed.

Probe-based confocal laser endomicroscopy acquisition protocol

During a pCLE acquisition protocol, the endoscopist typically inserts, through the working channel of a standard endoscope, a confocal miniprobe (Coloflex UHD, Cellvizio GI) of external diameter 2.5 mm, which is made of 30 000 optical fibers bundled together. The pCLE imaging setup, shown in Figure 1, allows the acquisition of pCLE images of field-of-view 240 μm at a rate of 9 to 12 frames per second. In stable pCLE video sequences, the probe is in constant contact with the tissue. Representative endoscopic, pCLE, and histopathology images of tubular adenoma are shown in Figure 1.

Prior to pCLE evaluation of the study polyps, the 2 expert endoscopists (Wallace MB, Buchner AM) viewed extensive published material on pCLE and performed a self-calibration on training pCLE videos of 20 polyps of known pathology (10 neoplastic and 10 non-neoplastic). These "training" polyps were evaluated by a gastrointestinal pathologist (Krishna M) and came from 9 patients not included in the study. Once acquired, the pCLE videos of the study lesions were evaluated off-line and in random order by the 2 expert endoscopists, who were blinded to histology diagnosis and endoscopic appearance of the lesion. The off-line diagnosis of pCLE videos was made based on the established modified Mainz criteria[11] for diagnosis of colorectal neoplasia, and according to pit pattern and overall crypt and vessel architecture. Of the whole pCLE video imaging of a polyp, the sequence of the video containing the most malignant pCLE features was considered to represent the polyp.

Histopathology as criterion standard diagnosis

All resected specimens were reviewed by a reference gastrointestinal pathologist (Krishna M) blinded to the pCLE information. Only the size and anatomic location were provided, which is the routine clinical practice at the Mayo Clinic institution. Intraepithelial neoplasia were defined using modified Vienna criteria[12,13]: benign polyps and hyperplastic polyps were classified as non-neoplastic lesions, while tubular adenoma, villous adenoma, tubulovillous adenoma and adenocarcinoma were classified as neoplastic lesions.

Standard bag-of-visual-words technique for content-based image retrieval

As endoscopists use perceptual similarities between pCLE videos of known diagnosis to establish a diagnosis on a new pCLE video, we propose a content-based retrieval approach to design the automated pCLE

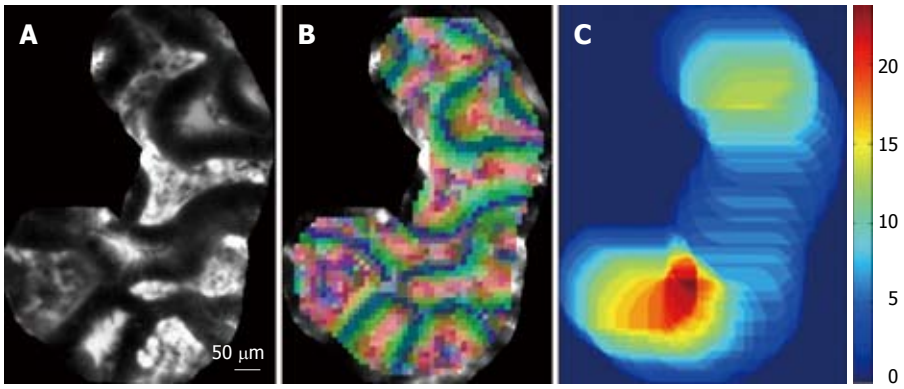


Figure 2 Adjusting bag-of-visual-words technique for probe-based confocal laser endomicroscopy video retrieval. A: Neoplastic probe-based confocal laser endomicroscopy mosaic obtained with non-rigid registration; B: Colored visual words mapped to the disk regions of radius 60 pixels in the mosaic image; C: Overlap scores of the local regions in the mosaic space, computed from the translation results of mosaicing.

video classification software. We revisited the standard bag-of-visual-words (BoW) technique which has been successfully used in many content-based image retrieval applications in computer vision[14]. A thorough technical presentation of our methodology has been disclosed previously[15], but without detailed clinical evaluation.

The standard BoW technique for image retrieval can be divided into four steps: region detection on the image, description of the regions, discretization of the feature space and similarity measuring between images. The detection step extracts salient regions in the image using sparse detectors. During the description step, a descriptor computes for each salient region its description vector. Then, the discretization step uses the result of a clustering method that builds K clusters, i.e., K visual words, from the union of the description vector sets gathered across all the images of the training database. Each description vector counts for one visual word, so an image can be represented by a signature of size K which is the histogram of its visual words. By construction, image signatures are invariant by viewpoint changes (image translation, rotation and scaling) and affine illumination changes. Finally, the similarity measuring step defines the similarity distance between two images as an adequate distance between their signatures: the most similar training images to the image of interest are defined as being the closest ones in terms of this distance.

Adjusting bag-of-visual-words technique for probe-based confocal laser endomicroscopy video retrieval

First, we observed that discriminative information is densely distributed in pCLE images. Second, we noticed that several pCLE image patterns have the same shape but represent different objects characterized by their different size (e.g., mesoscopic crypts and microscopic goblet cells both have a rounded shape). Therefore, pCLE image description must not be invariant by scaling. To avoid scale invariance and to extract all the image information, we decided to apply, instead of standard sparse detectors, a dense detector that was made of overlapping disks having a fixed radius and localized on a dense regular grid. We maintained the invariance by in-plane translation and rotation, because the pCLE miniprobe translates and rotates along the tissue surface. Besides, as the diffusion rate of fluorescein administered before imaging procedure decreases through time, invariance by affine illumination changes is also preserved.

Expert endoscopists pointed out that the field-of-view of single still images may not be large enough to make a robust diagnosis. Thus, we decided to retrieve not single images but complete videos, using the video mosaicing technique[16,17] (available in the Cellvizio software) to include spatial overlap between time-related images. Examples of mosaics built with the video mosaicing tool are shown in Figure 1. To ensure on-line retrieval, we use the translation results of the real-time version of the video-mosaicing technique to weight the contribution of each local image region to its visual word, as illustrated in Figure 2. Then, we computed the video signatures with a histogram summation technique. The whole pipeline of our retrieval-based software classification framework can be run on-line during ongoing colonoscopy (Figure 3).

Classification of probe-based confocal laser endomicroscopy videos using similarity distance

Once the visual signature of the video query is computed, the k-Nearest Neighbor search step identifies the k closest training videos to the video query, by relying on the similarity distance between the video signatures. We then used the known histopathology diagnosis of these training videos to classify the query video, either as neoplastic or as non-neoplastic. Each of the k most similar training videos delivers a "histopathological" vote which is weighted by the inverse of its similarity distance to the video query.

Due to the relatively small size of our pCLE database, we needed to learn from as much data as possible. To avoid any bias while having a large training set, we employed cross-validation. As several videos were acquired for the same patient, we performed a leave-one-patient-out cross-validation[18]: all videos from a given patient were excluded from the training set before being tested as queries of our retrieval and classification software. Cross-validation also allowed us to find the optimal number of nearest neighbors, $k = 9$, which is the one that maximizes the accuracy of the retrieval-based software classification results.

Statistical analysis

All the reported results of the automated software classi-

Table 1 Study population characteristics

Study population	Summary (n = 71)
Age (yr), median (min, 25th, 75th, max)	75 (46, 68, 79, 93)
Gender, %	
Male	49
Female	51
History of colon cancer, %	9
Family history of colon cancer, %	10

Table 2 Colorectal lesion characteristics

Colorectal lesions	Summary (n = 135)
Polyp size (mm), median (min, 25th, 75th, max)	8 (1, 5, 20, 60)
Polyp location, %	
Cecum	24
Rectum	20
Ascending	18
Sigmoid	14.5
Transverse	15
Descending	5.5
Splenic flex	3
Histopathology diagnosis, %	
Hyperplastic	31
Tubular adenoma	52
Tubulovillous adenoma	11.5
Hyperplastic and adenomatous features	2.5
Adenocarcinoma	3
Neoplastic lesion, simplified histopathology, %	69
Paris classification, %	
1p	1
1s	57
2a	32
2b	5
2c	1
2a/c	4

fication were obtained using leave-one-patient-out cross-validation. Statistical analysis was performed by André B.

To test for statistical difference between the two methods of interest, namely automated software classification and off-line classification by expert endoscopists, we used McNemar's tests[19] and showed the corresponding power calculations with a type I error alpha = 0.05. Two-sided P values < 0.05 were assumed to indicate statistical significance.

In order to assess statistical equivalence between the two methods, we used the two-sided Z-test between proportions[19,20] and computed 95% CI. Because the 135 pCLE videos constituted a small sample size, we used a correction for continuity for McNemar's test.

The statistics on overall accuracy are dependent on the relative fraction of non-neoplastic and neoplastic lesions examined, which in this study were 31.1% and 68.9%, respectively. Even though observations were made for more than one polyp in some patients, for the purposes of statistical analysis, individual polyps (and their corresponding videos) were assumed to constitute independent observations. It is recognized that there was multiple testing of outcome data arising from individual polyps. Since the statistical tests were meant to highlight differences, and since the correction by Bonferroni's method did not affect statistical significance in any of the comparisons, all P values are presented uncorrected for multiple testing.

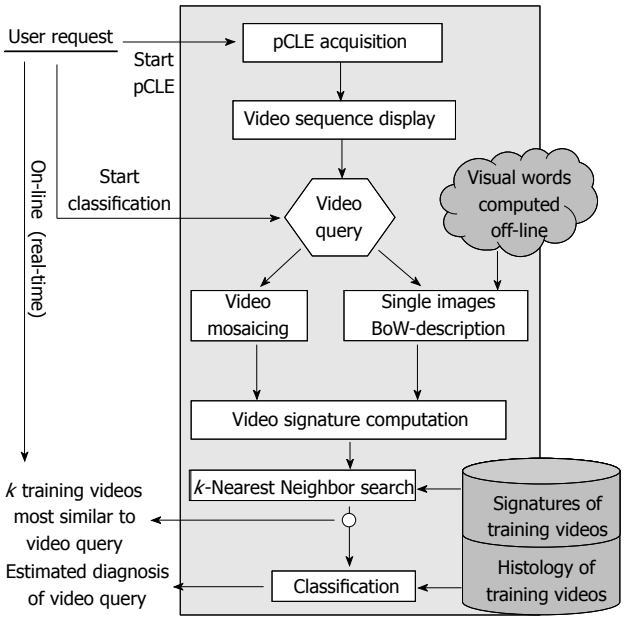


Figure 3 Pipeline of the probe-based confocal laser endomicroscopy retrieval-based software classification framework. From the acquisition of the probe-based confocal laser endomicroscopy (pCLE) video query by the Cellvizio system to the on-line automated diagnosis estimation.

RESULTS

Study population and colorectal lesion characteristics

Table 1 summarizes the demographic and general characteristics of the study population. None of the 71 patients experienced any endoscopic complications or adverse reactions to sodium fluorescein, with the exception of transient yellow discoloration of the skin and urine, which resolved by the time of discharge from the recovery room (skin) or within 24 h (urine). Histopathology and morphological classification of the 135 analyzed colorectal lesions are shown in Table 2.

Qualitative results of visual similarities between probe-based confocal laser endomicroscopy videos

The pCLE database contained 135 pCLE videos representing each of the 135 polyps. The pCLE appearance of neoplastic lesions, compared to that of non-neoplastic lesions, included dilated irregular vessels, fluorescein leakage, cellular features of epithelial mucin depletion, and histological features of villiform crypts with increased optical density along the epithelial border.

As the automated pCLE classification software is a similarity-based system that classified pCLE videos based on the votes of visually similar videos, its clinical relevance can be qualitatively evaluated by examining the intermediate results of video retrieval. Examples of mosaics built with the video mosaicing tool are shown in

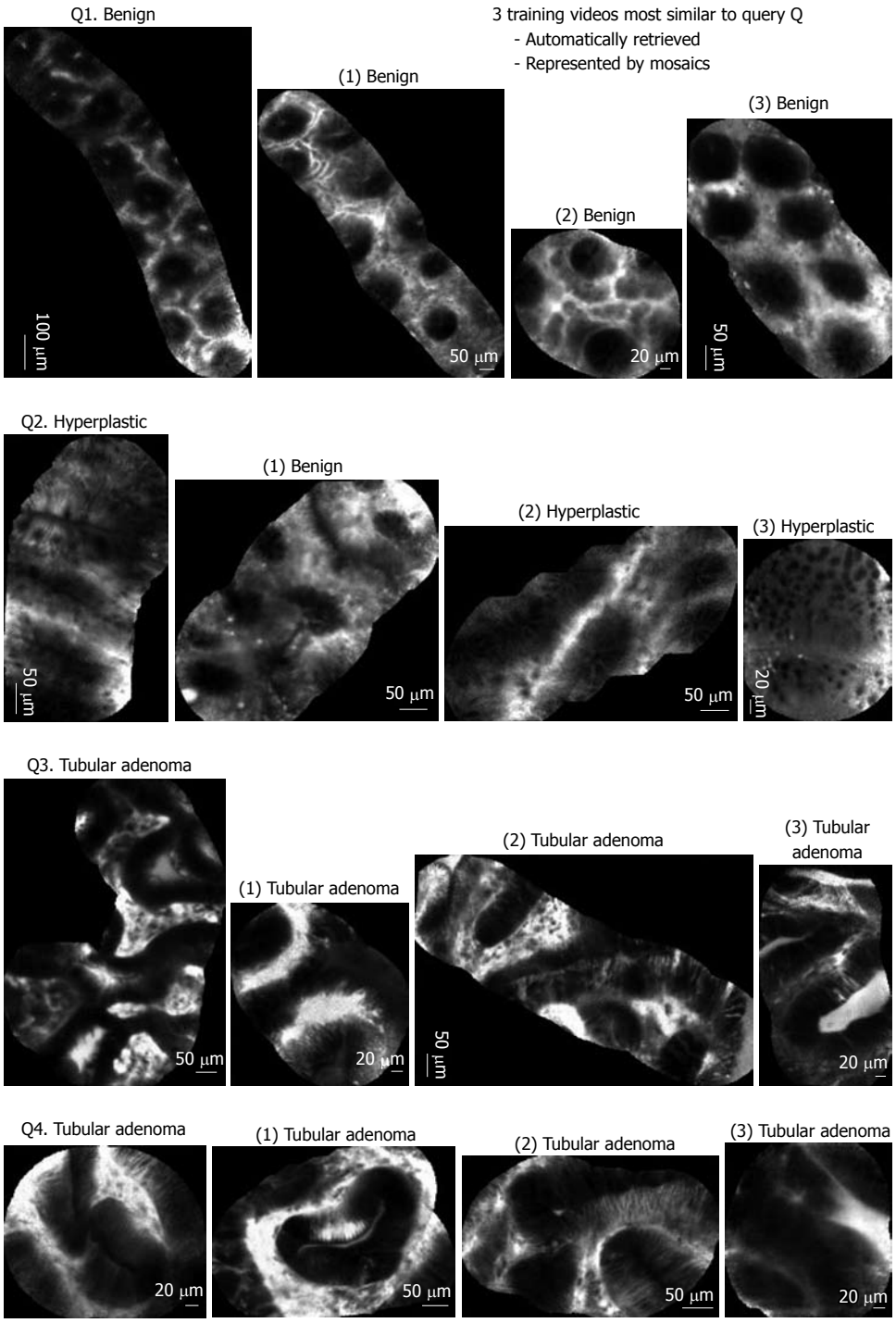


Figure 4 Typical results of automated probe-based confocal laser endomicroscopy video retrieval. The probe-based confocal laser endomicroscopy (pCLE) videos are represented by mosaic images; they are annotated with their histopathology diagnosis. Video queries are highlighted in gray and followed by their 3 most similar videos. Automated software classification (hyperplastic vs neoplastic) of query videos is based on the votes of the similar videos. With respect to histopathology, both the automated software classification and the pCLE diagnosis established by expert endoscopists are correct for these queries.

Figures 4 and 5. Figure 4 shows 5 typical results of the automated pCLE retrieval software. We observed that, despite the high variability in appearance of a given histopathological class (neoplastic or non-neoplastic), the automatically retrieved videos called "neighbors" looked quite similar to the video queries (Q1, Q2, Q3 and Q4, respectively). In addition, we noticed that the closer the neighbor was to the query, the more similar it was to it.

In terms of classification, the pathological class was estimated by the weighted votes of the 3 retrieved neighbors. In Figure 4, video queries Q1, Q2, Q3 and Q4 have been correctly classified with respect to histopathology, both by automated software classification and by expert endoscopists.

Figure 5 shows 3 other results that revealed some limitations of the automated pCLE retrieval software. Video query Q5 corresponds to a rare variety of hyperplastic polyp correctly classified as non-neoplastic by the expert endoscopists, but misclassified by the automated software classification because it was not represented in the training database for retrieval. Video query Q6 corresponds to the ambiguous serrated adenoma case, correctly classified as non-neoplastic by the automated software classification, but misclassified by the expert endoscopists who considered serrated adenomas as malignant. Video query Q7 corresponds to a tubulovillous

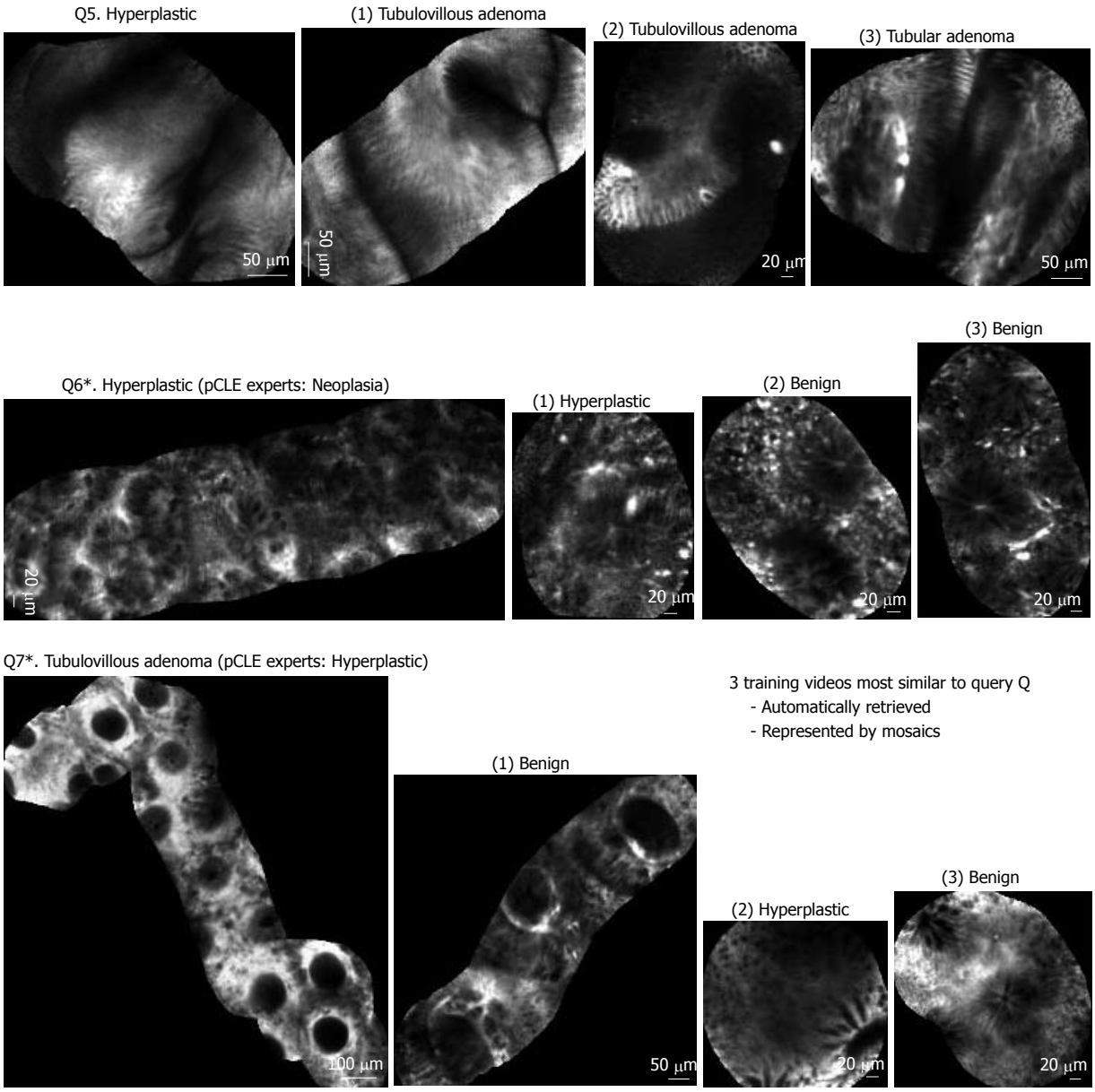


Figure 5 Results of automated probe-based confocal laser endomicroscopy video retrieval represented as mosaics. With respect to histology: the automated software classification is correct for video query Q6 but incorrect for video queries Q5 and Q7, whereas the off-line diagnosis of probe-based confocal laser endomicroscopy videos established by the expert endoscopists is correct for video queries Q5 but incorrect for video queries Q6 and Q7 (for which this disagreement is marked by *).

adenoma misclassified as non-neoplastic both by the expert endoscopists and by the automated software classification (this may be explained if a sampling error occurred and the corresponding biopsy was not performed exactly on the imaging spot).

Quantitative results of automated pCLE classification compared to off-line diagnosis of pCLE videos established by experts

Classification accuracy, sensitivity and specificity of the two methods, automated pCLE software classification (first method) and off-line diagnosis of pCLE videos established by the 2 expert endoscopists (second method), are listed in Table 3. Automated software classification reached a sensitivity of 92.5%, a specificity of 83.3% for a resulting accuracy of 89.6%. Expert review reached a sensitivity of 91.4%, a specificity of 85.7% and the same accuracy of 89.6%.

When testing for statistical difference, the *P* values provided by McNemar's tests showed that the differences between the 2 methods were not statistically significant and that there was very low power (< 6%) to detect the observed differences.

When testing for statistical equivalence, the 95% confidence intervals provided by two-sided *Z*-tests between proportions were: -0.073 to 0.073 for accuracy, -0.068 to 0.089 for sensitivity and -0.18 to 0.13 for specificity. These intervals included zero and were sufficiently small to suggest that the methods were equivalent. In particular, the -0.18 lower bound for specificity was acceptable

Table 3 Performance comparison between automated probe-based confocal laser endomicroscopy classification and off-line expert diagnosis of probe-based confocal laser endomicroscopy, for the differentiation between neoplastic and non-neoplastic colonic polyps

	Automated pCLE classification	Off-line expert diagnosis of pCLE
Accuracy		
%	89.6	89.6
Fraction	121/135	121/135
Sensitivity		
%	92.5	91.4
Fraction	86/93	85/93
Specificity		
%	83.3	85.7
Fraction	35/42	36/42
Statistical significance between (1) and (2)		
McNemar's test, alpha = 0.05		
Accuracy: (*P*, power)	(Not significant, 2.5%)	
Sensitivity: (*P*, power)	(Not significant, 6.5%)	
Specificity: (*P*, power)	(Not significant, 5.2%)	
Statistical equivalence between (1) and (2)		
Two-sided *Z* test		
95% CI for accuracy	-0.073-0.073	
95% CI for sensitivity	-0.068-0.089	
95% CI for specificity	-0.18-0.13	

pCLE: Probe-based confocal laser endomicroscopy.

if the automated pCLE classification software was only taken as a second-reader tool to support pCLE diagnosis.

DISCUSSION

The present study demonstrates that, using a fairly representative database of colonic polyps, our automated software for the pCLE video classification has overall high accuracy, sensitivity and specificity, that are comparable to those of the off-line diagnosis of pCLE videos established by two endoscopists expert in pCLE. As the automated classification software can be run on-line during ongoing colonoscopy, it could be used as a second-reader tool to support and improve not only off-line but also on-line diagnosis of pCLE established by endoscopists with various levels of expertise. In the majority of cases, the second reader would agree with a moderately experienced endoscopist, who would thus be comforted in his/her diagnosis. For cases when they disagree, the endoscopist would have the opportunity to rethink his/her diagnosis and have more accurate *in vivo* interpretation. Besides, especially for small polyps, this second-reader tool could assist the endoscopist in adopting the "Diagnose, Resect and Discard Strategy" that dispenses with histopathological examination.

Gomez *et al*[21] analyzed *in vivo* pCLE interpretation in distinguishing between neoplastic and non-neoplastic lesions among 3 expert endoscopists and estimated an average accuracy of 75% (sensitivity 76%, specificity 72%) with good to moderate interobserver agreement. Buchner *et al*[22] demonstrated that accurate interpretation of pCLE images by 11 endoscopists, considered as non-expert in pCLE, can be learned rapidly with a short 2-h training session. The learning curve pattern of pCLE in predicting neoplastic lesions was demonstrated with improved accuracies in time from 63% to 86% as observers' experience increased. Thus, prospectively, the automated classification software could be valuable not only for *in vivo* diagnosis support, but also for training support to improve the learning curve of new endoscopists. Indeed, we have shown in a preliminary study[23] how interpretation difficulty can be automatically estimated by the software, in order to develop a self-training simulator for pCLE diagnosis with adjustable level of difficulty. For surgical skills, evidence of the learning effect from the use of training tools have been provided in the thesis by Brydges[24], however, further investigation is needed for the extension of learning effect analysis to diagnostic skills.

One of the advantages of our classification software is that it is not a "black box" but an informative tool based on the query by example model: it produces, as intermediate results, visually similar annotated videos that are directly interpretable by the endoscopist. From the qualitative observations of visual similarities between pCLE videos, we suggest that the visually convincing results of the intermediate video retrieval step account for the relevance of the whole pCLE classification software. As few similar videos (less than 10) are necessary to classify a video query with high accuracy, this visual information should be clinically useful for the endoscopist.

Further limitations of the classification software may include three main issues. First, a large training database is needed to be sufficiently representative of non-typical pCLE cases. This is even more challenging since the practice of pCLE is evolving and that new cases with atypical pCLE features may still be encountered. Second, the definition of "criterion standard" for colorectal cancer screening is debatable because expert endoscopists and pathologists do not always agree. This can be illustrated by many examples of hyperplastic polyps redefined later as sessile serrated lesions by gastrointestinal pathologists, as in the study by Khalid *et al*[25]. The third limitation is that an obtained biopsy may be acquired unintentionally from the area that does not correspond with the obtained pCLE imaging.

The task of the automated pCLE classification software is not to replace the endoscopist or the pathologist but to assist the endoscopist in making an informed decision. Before using the computer-based classification tool during an ongoing endoscopy procedure, more work is needed to improve its accuracy and to develop underlying tools that are both ergonomic and complementary. In particular, the on-line display of the retrieval outputs, for instance of the 3 most similar videos to the video query, together with their histopathology and possible multimodal clinical data, may be a precious underlying indicator for diagnosis decision. Such a sophisticated "Smart Atlas" for pCLE would allow endoscopists in different centers to share and enrich their pCLE knowl-

edge during ongoing endoscopy. Further studies are warranted to evaluate the impact of using automated pCLE retrieval and classification software on the pCLE learning curve and on the diagnostic performance of the endoscopists.

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COMMENTS

Background

Histopathology is the criterion standard for the diagnosis of colorectal cancers, but it implies a large proportion of unnecessary polypectomies and an inherent delay in diagnosis. Probe-based confocal laser endomicroscopy (pCLE) is a recent technology that enables, during ongoing endoscopy, *in vivo* imaging of the epithelium at the microscopic level.

Research frontiers

Several studies have already demonstrated the applicability of pCLE in diagnosing colorectal neoplasia *in vivo* with high sensitivity and specificity. Because pCLE is a relatively recent imaging technology, the interpretation of pCLE videos of colonic polyps for diagnostic purposes is still challenging for many non-expert endoscopists.

Innovations and breakthroughs

This is believed to be the first study to propose, with the aim of supporting *in vivo* diagnosis of colorectal cancers, content-based image retrieval-based classification software that automatically extracts visually similar annotated videos directly interpretable by the endoscopist. The extracted annotated videos can be presented to the endoscopist in a second reader paradigm to better support pCLE diagnosis. Furthermore, this study demonstrates that this novel software achieves a high diagnostic performance, which is statistically comparable to that of off-line diagnosis of pCLE videos established by expert endoscopists.

Applications

The classification software proposed in this study is an objective tool which has the potential to support the interpretation of pCLE videos of colonic polyps for diagnostic purposes. Further studies are warranted to evaluate the impact of using the automated classification software on the pCLE learning curve and on the diagnostic performance of endoscopists.

Terminology

pCLE: An imaging system that allows the endoscopist to visualize the epithelium *in vivo*, at the microscopic level and in real-time during ongoing endoscopy; Content-based image retrieval: A computer vision technique that automatically extracts, given a query image, several training images with the most similar appearance to the query.

Peer review

This is a good descriptive study in which authors support probe-based confocal laser endomicroscopy diagnosis by designing a software for automated classification of colonic polyps. The results are interesting and suggest that the proposed software for automated classification of pCLE videos of colonic polyps achieves high performance, comparable to that of off-line diagnosis of pCLE videos established by expert endoscopists.

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BRIEF ARTICLE

Quantitation of HBsAg predicts response to entecavir therapy in HBV genotype C patients

Etsuro Orito, Kei Fujiwara, Hiroshi Kanie, Tesshin Ban, Tomonori Yamada, Katsumi Hayashi

Etsuro Orito, Kei Fujiwara, Hiroshi Kanie, Tesshin Ban, Tomonori Yamada, Katsumi Hayashi, Department of Gastroenterology, Nagoya Daini Red Cross Hospital, Nagoya 466-8650, Japan
Author contributions: Orito E, Fujiwara K, Kanie H, Ban T, Yamada T and Hayashi K designed the study, enrolled the patients, analyzed the data, and drafted the manuscript; and Ban T contributed to the statistical analysis.
Supported by A grant from the Japanese Ministry of Health and Welfare
Correspondence to: Etsuro Orito, MD, PhD, Department of Gastroenterology, Nagoya Daini Red Cross Hospital, Myoken-cho 2-9, Showa, Nagoya 466-8650, Japan. orito@nagoya2.jrc.or.jp
Telephone: +81-52-8321121 Fax: +81-52-8325369
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Abstract

AIM: To analysis the factors that predict the response to entecavir therapy in chronic hepatitis patients with hepatitis B virus (HBV) genotype C.

METHODS: Fifty patients [hepatitis B e antigen (HBeAg)-negative:HBeAg-positive = 26:24] with HBV genotype C, who received naïve entecavir therapy for > 2 years, were analyzed. Patients who showed HBV DNA levels ⩾ 3.0 log viral copies/mL after 2 years of entecavir therapy were designated as slow-responders, while those that showed < 3.0 log copies/mL were termed rapid-responders. Quantitative hepatitis B surface antigen (HBsAg) levels (qHBsAg) were determined by the Architect HBsAg QT immunoassay. Hepatitis B core-related antigen was detected by enzyme immunoassay. Pre-C and Core promoter mutations were determined using by polymerase chain reaction (PCR). Drug-resistance mutations were detected by the PCR-Invader method.

RESULTS: At year 2, HBV DNA levels in all patients in the HBeAg-negative group were < 3.0 log copies/mL. In contrast, in the HBeAg-positive group, 41.7% were slow-responders, while 58.3% were rapid-responders. No entecavir-resistant mutants were detected in the slow-responders. When the pretreatment factors were compared between the slow- and rapid-responders; the median qHBsAg in the slow-responders was 4.57 log IU/mL, compared with 3.63 log IU/mL in the rapid-responders (P < 0.01). When the pretreatment factors predictive of HBV DNA-negative status at year 2 in all 50 patients were analyzed, HBeAg-negative status, low HBV DNA levels, and low qHBsAg levels were significant (P < 0.01). Multivariate analysis revealed that the low qHBsAg level was the most significant predictive factor (P = 0.03).

CONCLUSION: Quantitation of HBsAg could be a useful indicator to predict response to entecavir therapy.

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Key words: Chronic hepatitis B; Quantitation of hepatitis B surface antigen; Entecavir; Hepatitis B virus genotype C; Slow-responders; Hepatitis B core-related antigen; Core promoter mutation; Pre-C mutation

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INTRODUCTION

Hepatitis B virus (HBV) is a major causative agent of chronic liver diseases[1]. Various strains of HBV have been isolated all over the world, and have been classified as HBV genotypes from A to J[2]. In Japan, about 85% of patients have HBV genotype C, and about 12% have HBV genotype B[3]. Worldwide, HBV genotypes show specific geographical distributions. HBV genotypes A and D are prevalent in the United States and Europe, while HBV genotypes B and C are prevalent in Asia[4]. Disease progression and prevalence of hepatitis B e antigen (HBeAg)-positive status are often associated with HBV genotypes[5]. Therefore, we analyzed the clinical and virological features of patients with HBV genotype C and homogenous backgrounds, because HBV genotype C is the predominant type in Japan.

Entecavir is widely used as a first-choice nucleot(s)ide analog (NA) for chronic hepatitis B patients, because less than 1% of entecavir-naive patients developed resistant mutants after 5 years of therapy[6-8]. However, in HBeAg-positive patients, the response rate to entecavir therapy is less favorable compared with HBeAg-negative patients[6,7]. In addition, some patients show a slow-response, which indicates that serum HBV DNA levels remain high after long-term entecavir therapy. However, it is unclear which patients become slow-responders. Therefore, the aim of this study is to clarify the virological and clinical characteristics of the slow-responders before and during long-term entecavir therapy for HBV genotype C.

MATERIALS AND METHODS

Patient population

From July 2007, 102 consecutive hepatitis B surface antigen (HBsAg)-positive patients with chronic liver disease were enrolled in a naïve entecavir therapy in our hospital. Ten patients dropped out, 15 patients discontinued therapy, 10 patients received immunosuppressive therapy during entecavir therapy, and 10 patients received entecavir for less than 2 years. Thus, 57 patients were analyzed in this prospective, single center study. The institutional review board of the hospital approved the study. Serum samples were drawn from the patients after obtaining written informed consent.

All the patients received 0.5 mg of entecavir daily. Patients with poor adherence were excluded from the study.

All patients were positive for HBsAg for more than 6 mo, had serum HBV DNA of ≥ 3 log viral copies/mL, were negative for anti-HCV, and were negative for anti-human immunodeficiency virus before entecavir therapy. Patients with decompensated cirrhosis, acute hepatitis, or acute exacerbation were excluded. Liver biopsy was not performed in some patients; therefore, the liver disease status was diagnosed by the clinical, laboratory, and imaging tests.

HBeAg-positive patients whose serum HBV DNA levels remained ≥ 3.0 log copies/mL after 2 years of entecavir therapy were considered to be slow-responders, while patients with < 3.0 log copies/mL were designated as rapid-responders.

Table 1 Baseline characteristics of the patients with hepatitis B virus genotype C

	HBeAg-negative group	HBeAg-positive group	*P* value
No.	26	24	NS
Age (yr)	57.2 (35-80)	44.2 (35-71)	< 0.01
Gender, M:F	15:16	16:11	NS
Diseases, CH:LC/HCC	21:5	23:1	NS
ALT (IU/mL)	38 (13-950)	102 (812-602)	< 0.01
Platelet counts ($\times 10^4$/mL)	18.6 (3.4-4.9)	18.0 (8.4-26.8)	NS
Albumin (mg/dL)	4.3 (3.4-4.9)	4.2 (2.3-5.0)	NS
Serum HBV DNA level (log copies/mL)	5.1 (3.9-8.8)	7.6 (5.6-8.8)	< 0.01

All data are shown by median (range). HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; NS: Not significant.

Laboratory tests

Quantitation of HBsAg (qHBsAg) was performed using the Architect HBsAg QT immunoassay (Abott Japan, Tokyo, Japan), in accordance with the manufacturer's instructions[9]. The detection range was 0.05 to 250 IU/mL. If HBsAg levels were found to be higher than 250 IU/mL, samples were diluted to 1:500 to 1:20 000. In this study, the results of the quantitative HBsAg levels are shown as the logarithmic value. HBV genotypes were detected by enzyme immunoassay (EIA) (Institute of Immunology, Tokyo, Japan)[10]. The HBV core-related antigen (HBcrAg) was detected by a chemilimunescent EIA method (Fujirebio Inc., Tokyo, Japan)[11]. Pre-C mutation and Core promoter mutations were detected by polymerase chain reaction (PCR) (HBV DNA precore/core promoter mutation decision kit; Roche Diagnostics Japan, Tokyo, Japan). Drug-resistant mutations in HBV against nucleotide analogs (NAs; lamivudine, adefovir and entecavir) were detected by the PCR-Invader method (BML Inc., Tokyo, Japan)[12].

RESULTS

Of the 57 patients, 50 patients were genotype C, three patients were HBV genotype A, one was genotype D, and three were of indeterminate genotype on EIA. Thus, the 50 patients with HBV genotype C were analyzed. Baseline characteristics of the 50 patients are shown in Table 1. The median age of the HBeAg-negative group age was significantly higher, the median alanine aminotransferase (ALT) level was significantly lower, and the median HBV DNA level was significantly lower than those in the HBeAg-positive group.

After 2 years of entecavir therapy, the rates of normalization (< 40 IU/L) of ALT levels were 87.0% in the HBeAg-negative group and 92.5% in the HBeAg-positive group (*P* = Not significant).

In contrast, at year 2, the rates of reduction in HBV

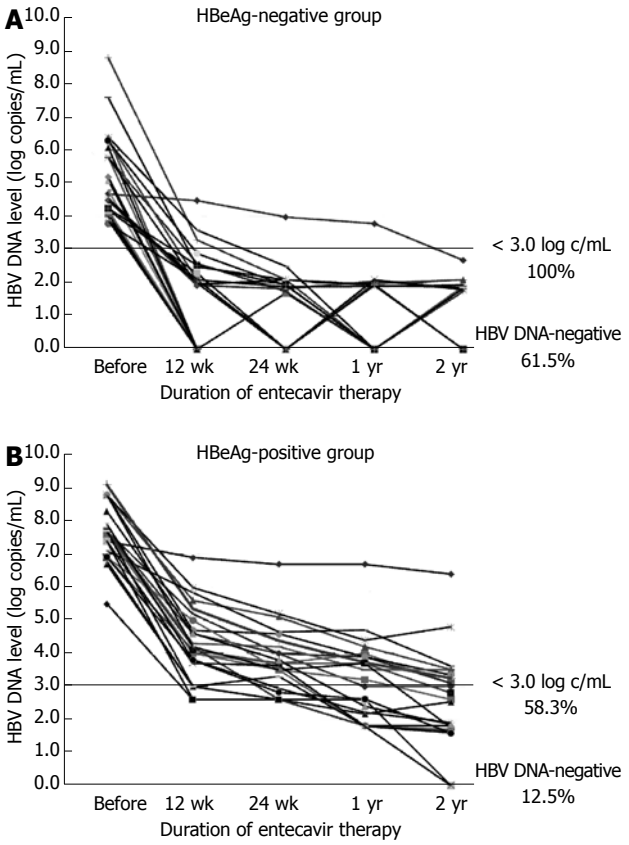


Figure 1 Hepatitis B virus DNA levels before and during entecavir therapy. A: In the hepatitis B e antigen (HBeAg)-negative group, hepatitis B virus (HBV) DNA levels in all patients decreased to < 3.0 log copies/mL at year 2. Of these patients, 61.5% shown to be negative for HBV DNA by the real-time polymerase chain reaction method; B: In the HBeAg-positive group, 58.3% of patients (rapid-responders) showed < 3.0 log copies/mL at year 2, while 41.7% of patients (slow-responders) showed ≥ 3.0 log copies/mL. In addition, at year 2, only 12.5% of the patients were negative for HBV DNA.

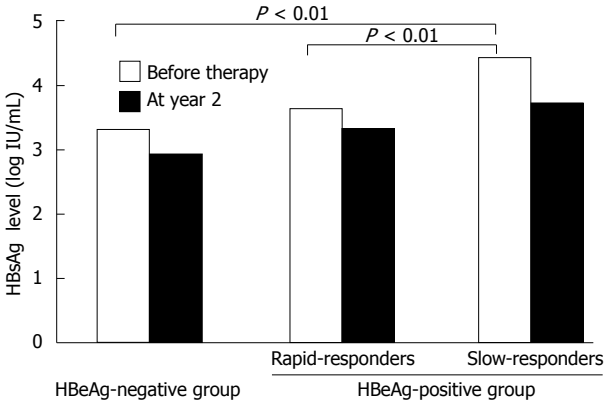


Figure 2 Median quantitative hepatitis B surface antigen levels among patients receiving entecavir therapy. Quantitative hepatitis B surface antigen (HBsAg) levels (qHBsAg) levels in slow-responders in the HBeAg-positive group were significantly higher than those in rapid-responders and the hepatitis B e antigen (HBeAg)-negative group. The median qHBsAg level at year 2 in the slow-responders remained higher than in other groups.

DNA to < 3.0 log copies/mL were 100% in the HBeAg-negative group and 58.3% in the HBeAg-positive group ($P < 0.01$). Thus, in the HBeAg-positive group, 58.3% of patients were designated as rapid-responders, and 41.7% were designated as slow-responders (HBV DNA levels ≥ 3.0 log copies/mL at year 2) (Figure 1). In addition, in the HBeAg-negative group, real-time PCR indicated that 61.5% of the patients were negative for HBV DNA, compared to 12.5% of the HBeAg-positive patients ($P < 0.01$).

Baseline data

When pre-treatment factors were compared between the rapid- and slow-responders (Table 2), age, gender, disease, platelet counts, and albumin were not significantly different. The median ALT level in the rapid-responders group was 131 IU/L compared with 31 IU/L in the slow-responders ($P = 0.02$). The pre-treatment median HBV DNA levels were 7.4 log copies/mL in the rapid-responders, and 8.3 in the slow-responders ($P = 0.06$). The was no difference in the rate of Pre-C and Core promoter mutations between the responder groups. In contrast, the rate of Pre-C mutations in the HBeAg-negative group was 83.3%, compared with 0% in the HBeAg-positive group. Pre-treatment HBcrAg levels did not differ among the three groups. In contrast, the pre-treatment median qHBsAg level was 3.63 log IU/mL in the rapid-responders, compared with 4.57 log IU/mL in the slow-responders ($P < 0.01$).

Data at year 2

At year 2 of therapy, the median qHBsAg level in the rapid-responders was 3.25 log IU/mL, compared with 4.12 log IU/mL in the slow-responders ($P = 0.01$). The median HBcrAg level in the rapid-responders was 5.85 log U/mL, compared with > 6.8 (the upper limit of the detection range) in the slow-responders ($P < 0.01$). In Figure 2, qHBsAg levels before treatment and at year 2 are shown for the HBeAg-negative group, and the rapid-responder and slow-responders in the HBeAg-positive group.

Among all the slow-responders, no entecavir-resistant mutations were found, although three patients showed M204I lamivudine-resistant mutations (Table 3).

Comparison between HBV DNA-negative and -positive patients at year 2 during entecavir therapy in all the patients

At year 2 of entecavir therapy, among 50 patients, real-time PCR showed that 19 (38.0%) were negative for HBV DNA, compared with 31 (62.0%) who were still positive for HBV DNA (Table 4). The pretreatment clinical and virological characteristics between the HBV DNA-negative and -positive groups were compared by univariate analysis. In the HBV DNA-negative group, the median ALT level was significantly lower, the rate of HBeAg-negative status was significantly higher, the median HBV DNA level was lower, and the median qHBsAg level was lower, than those in the HBV DNA-positive group.

However, when multivariate analysis using logistic regression analysis was performed, the median qHBsAg level was the only significant factor that predicted the negative HBV DNA status at year 2 of entecavir therapy

Table 2 Clinical and virological results among the hepatitis B e antigen-negative group, the rapid-responders, and the slow-responders in the hepatitis B e antigen-positive group during 2 years of entecavir therapy

Characteristics	HBeAg-negative group	HBeAg-positive group		*P* value
	(*n* = 26)	RR (*n* = 14)	SR (*n* = 10)	RR *vs* SR
<Baseline data>				
Age	58 (35-80)	45 (34-68)	43 (31-71)	NS
Gender (male:female)	13:13	9:5	6:4	NS
Disease (CH:LC/HCC)	21:5	13:1	6:4	NS
ALT (IU/L)	38 (13-950)	131 (12-602)	31 (13-108)	0.02
Platelet count ($\times 10^4$/mL)	18.6 (3.4-35.1)	17.1 (8.4-22.4)	20.0 (11.0-26.8)	NS
Albumin (mg/dL)	4.3 (3.4-4.9)	4.0 (2.3-5.0)	4.4 (3.7-4.6)	NS
HBV genotype C	100%	100%	100%	NS
HBV DNA (log copies/mL)	5.1 (3.9-8.8)	7.4 (5.6-8.8)	8.3 (7.1-8.8)	NS
qHBsAg (log IU/mL)	3.17 (0.70-4.58)	3.63 (1.68-4.34)	4.57 (4.35-4.76)	< 0.01
HBcrAg (log U/mL)	3.6 (3.0-> 6.8)	> 6.8 (6-> 6.8)	> 6.8 (> 6.8-> 6.8)	NS
Pre-C mutation (%)	83.3	0	0	NS
Core promoter mutation (%)	58.3	57.1	50.0	NS
<At year 2 during therapy>				
HBV DNA (log copies/mL)	0.0 (0.0-2.7)	2.1 (0.0-2.1)	3.5 (3.1-6.9)	-
ALT (IU/L)	18 (9-75)	17.5 (10-31)	23 (13-37)	NS
HBeAg seroconversion	-	23.50%	0%	NS
HBsAg seroclearance	0%	0%	0%	NS
qHBsAg (log IU/mL)	2.91 (0.62-3.9)	3.25 (1.70-3.92)	4.12 (3.23-4.47)	0.01
HBcrAg (log U/mL)	3.0 (3.0-5.4)	5.9 (4.0-> 6.8)	> 6.8 (5.2-> 6.8)	< 0.01
Resistant mutations against entecavir	UDL	UDL	0%	-

HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; UDL: Under the detection limit; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; qHBsAg: Quantitation of HBsAg level; HBcrAg: HBV core-related antigen; NS: Not significant; RR: Rapid-responder; SR: Slow-responder.

Table 3 Drug resistant mutations in the slow-responders at year 2

Patient	Age (yr)	Gender	Previous therapy	HBV genotype	Drug resistant mutations against						
					Lam L180	Lam M204	Lam/Ade A181	Ade N236	Ent T184	Ent S202	Ent M205
1	52	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
2	35	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
3	68	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
4	56	Female	No	C	Wild	M204I	Wild	Wild	Wild	Wild	Wild
5	36	Female	No	C	Wild	M204I	Wild	Wild	Wild	Wild	Wild
6	45	Male	No	C	Wild	M204I	Wild	Wild	Wild	Wild	Wild
7	35	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
8	67	Female	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
9	39	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
10	44	Female	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild

Lam: Lamivudine; Ade: Adefovir; Ent: Entecavir; HBV: Hepatitis B virus.

(odds ratio 8.16, 95% CI: 1.28-52.18, $P = 0.03$).

DISCUSSION

In this study, the clinical and virological features of patients with HBV genotype C who received naïve-entecavir therapy were analyzed. After 2 years of entecavir therapy, about 42% of the HBeAg-positive patients showed HBV DNA levels ≥ 3 log copies/mL, while all of the HBeAg-negative patients showed < 3 log copies/mL. Therefore, the factors associated with the slow response to entecavir therapy among the HBeAg-positive group were studied initially. In addition, among the 50 patients, 38% showed HBV DNA-negative status at year 2. Thus, the pretreatment factors that predict the loss of HBV DNA were analyzed in all 50 patients. According to the multivariate analysis, qHBsAg levels are the most important factor for predicting the response to entecavir therapy in patients with HBV genotype C.

In Japan, HBV genotype C is the most prevalent[3]. The response rates to interferon or NA therapy in patients with HBV genotype C, as well as D, are poor when compared to those with HBV genotype B or A[13]. Thus, in this study, only subjects with HBV genotype C were studied.

Recently, a decline in HBsAg levels during PEG-interferon therapy was reported to be significant in the evaluation of the response to therapy[14-17]. In these reports, HBsAg levels were found to be one of the best viral markers for predicting response to anti-viral therapy and viral

Table 4 Pretreatment clinical and virological characteristics between hepatitis B virus DNA-negative and -positive group at year 2 during entecavir therapy

Characteristics	HBV DNA-negative group (n = 19)	HBV DNA-positive group (n = 31)	P value	
			Univariate analysis	Multivariate analysis
Age	51 (31-73)	52 (32-80)	NS	
Gender (male:female)	12:7	20:11	NS	
Disease (CH:LC/HCC)	17:2	27:4	NS	
ALT (IU/L)	36 (12-366)	108 (13-602)	0.03	NS
Platelet counts ($\times 10^4$/mL)	19.0 (8.8-35.1)	17.8 (3.4-26.8)	NS	
Albumin (mg/dL)	4.35 (3.84-4.85)	4.14 (2.28-4.72)	NS	
HBV genotype (B:C:others)	0:19:0	0:31:0	NS	
HBeAg status (positive:negative)	3:16	21:10	< 0.01	NS
HBV DNA (log copies/mL)	5.1 (3.1-7.4)	7.6 (3.7-8.8)	< 0.01	NS
qHBsAg level (log IU/mL)	3.31 (1.90-4.08)	4.20 (3.06-4.87)	< 0.01	0.03
HBcrAg level (log U/mL)	3.45 (3.0-> 6.8)	> 6.8 (3.0-> 6.8)	NS	
Pre-C mutation (%)	75.0	43.3	NS	
Core promoter mutation (%)	37.5	60.0	NS	

CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; qHBsAg: Quantitation of hepatitis B surface antigen level; HBcrAg: HBV core-related antigen; NS: Not significant.

activity levels in hepatocytes, correlating with cccDNA levels[14,18]. Although qHBsAg is a predictor of response to entecavir therapy[19], there have been no reports in a homogeneous HBV genotype setting. In this study, qHBsAg was demonstrated to be the most significant predictor of entecavir therapy in patients with HBV genotype C.

HBV DNA levels are also considered an important factor associated with response to anti-viral therapy[17]. In this study, there was a tendency for higher HBV DNA levels in the slow-responders as compared to rapid-responders. The association between HBV DNA levels and response to therapy may be clarified further in a larger number of patients.

HBcrAg levels indicate the serum HB core antigen levels plus HBeAg levels[11]. HBcrAg levels are reported to be associated with cccDNA levels in hepatocytes[20]. However, this study showed no association between HBcrAg levels and slow response. This may be explained by the narrow quantitation range of HBcrAg levels, because HBcrAg levels in most patients in the HBeAg-positive group were greater than the upper level of the detection range of the assay.

The association between Core promoter mutation and response rate to NAs is also interesting, because the replication level of HBV is thought to be high in patients with Core promoter mutations. We reported previously that, in patients with HBV genotype C, the rates of HBeAg-positive status and Core promoter mutations are higher than those in patients with HBV genotype B[5]. In this study, a higher rate of Core promoter mutations was observed in the HBeAg-positive patients with HBV genotype C. In addition, a higher rate of Pre-C mutations was observed in the HBeAg-negative group. However, no association between the mutation rate and response rate to therapy was demonstrated in this study.

Higher ALT levels are considered an important factor in predicting good response to PEG-interferon therapy[21]. However, low ALT levels were observed in the HBV DNA-negative group at year 2 of therapy, because a high proportion of the HBeAg-negative patients had low ALT levels, compared to the HBeAg-positive patients. Thus, we consider that, during entecavir therapy, ALT levels are not associated with treatment response.

In this study, no resistant mutations against entecavir were found during 2 years therapy. As reported previously, resistant mutations against entecavir are rarely developed during 5 years of entecavir therapy[8]. Therefore, slow response was not caused by entecavir-resistant mutants.

In conclusion, we suggest that qHBsAg is a significant and convenient indicator for predicting response to entecavir therapy.

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COMMENTS

Background

Entecavir is a nucleot(s)ide analog that is widely used for the treatment of chronic hepatitis B patients. The efficacy of entecavir is very good for hepatitis B e antigen (HBeAg)-negative patients, but not so good for HBeAg-positive patients. The prognosis and response to anti-viral therapies depend on hepatitis B virus (HBV) genotype. The factors that affect the efficacy of entecavir therapy are still unclear, especially in patients with HBV genotype C.

Research frontiers

As quantitation assay of serum hepatitis B surface antigen (HBsAg) has been recently developed, allowing the serum level of HBsAg to be determined over a very wide range. The upper range of HBsAg levels could be detected to 6.7 log IU/mL by the Architect HBsAg QT immunoassay when samples were diluted to 1:20 000. Thus, the authors could analyze the relationship between the efficacy of entecavir therapy and various HBV markers.

Innovations and breakthroughs

This study showed that the quantitative HBsAg level is a significant factor for predicting the efficacy of entecavir therapy in patients with HBV genotype C. Patients with low levels of HBsAg before entecavir therapy often show HBV DNA levels < 3.0 log copies/mL or are negative for HBV DNA at year 2 during therapy.

Applications

Using the quantitation of HBsAg, the efficacy of various anti-viral therapies can be predicted before treatment. The quantitation of HBsAg could be a useful tool for determining the treatment schedule for chronic hepatitis B patients.

Peer review

Although small in patient numbers, the subject matter is interesting and original. This study adds to the emerging data suggesting HBsAg decline is a predictor of response.

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BRIEF ARTICLE

Inhibition of gastric perception of mild distention by omeprazole in volunteers

Akihito Iida, Hiroshi Kaneko, Toshihiro Konagaya, Yasushi Funaki, Kentaro Tokudome, Shinya Izawa, Yasuhiro Tamura, Mari Mizuno, Naotaka Ogasawara, Makoto Sasaki, Kunio Kasugai

Akihito Iida, Yasushi Funaki, Kentaro Tokudome, Shinya Izawa, Yasuhiro Tamura, Mari Mizuno, Naotaka Ogasawara, Makoto Sasaki, Kunio Kasugai, Division of Gastroenterology, Department of Internal Medicine, School of Medicine, Aichi Medical University, Aichi 480-1195, Japan
Hiroshi Kaneko, Department of Internal Medicine, Hoshigaoka Maternity Hospital, Aichi 464-0026, Japan
Toshihiro Konagaya, Marine Clinic, Nagoya, Aichi 460-0002, Japan
Author contributions: Iida A, Kaneko H, Konagaya T, Ogasawara N, Sasaki M, and Kasugai K designed the research; Iida A, Kaneko H, Konagaya T, Funaki Y, Tokudome K, Izawa S, Tamura Y, and Mizuno M performed the research; Iida A and Konagaya T analyzed the data; and Iida A and Kaneko H wrote the manuscript.
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Correspondence to: Akihito Iida, MD, PhD, Assistant Professor, Lecturer, Division of Gastroenterology, Department of Internal Medicine, School of Medicine, Aichi Medical University, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan. iida@aichi-med-u.ac.jp
Telephone: +81-561-623311 Fax: +81-561-621508
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Abstract

AIM: To evaluate the effects of omeprazole on gastric mechanosensitivity in humans.

METHODS: A double lumen polyvinyl tube with a plastic bag was introduced into the stomach of healthy volunteers under fluorography and connected to a barostat device. Subjects were then positioned so they were sitting comfortably, and the minimal distending pressure (MDP) was determined after a 30-min adaptation period. Isobaric distensions were performed in stepwise increments of 2 mmHg (2 min each) starting from the MDP. Subjects were instructed to score feelings at the end of every step using a graphic rating scale: 0, no perception; 1, weak/vague; 2, weak but significant; 3, moderate/vague; 4, moderate but significant; 5, severe discomfort; and 6, unbearable pain. After this first test, subjects received omeprazole (20 mg, after dinner) once daily for 1 wk. A second test was performed on the last day of treatment.

RESULTS: No adverse effects were observed. Mean MDP before and after treatment was 6.3 ± 0.3 mmHg and 6.2 ± 0.5 mmHg, respectively. One subject before and 2 after treatment did not reach a score of 6 at the maximum bag volume of 750 mL. After omeprazole, there was a significant increase in the distension pressure required to reach scores of 1 (P = 0.019) and 2 (P = 0.017) as compared to baseline. There were no changes in pressure required to reach the other scores after treatment. Two subjects before and one after omeprazole rated their abdominal feeling < 1 at MDP, and mean (± SE) abdominal discomfort scores at MDP were 0.13 ± 0.09 and 0.04 ± 0.04, respectively. Mean scores induced by each MDP + 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 (mmHg) were 1.1 ± 0.3, 2.0 ± 0.4, 2.9 ± 0.5, 3.3 ± 0.4, 4.6 ± 0.3, 5.2 ± 0.3, 5.5 ± 0.2, 5.5 ± 0.3, 5.7 ± 0.3, and 5.4, respectively. After omeprazole, abdominal feeling scores for the same incremental pressures over MDP were 0.3 ± 0.1, 0.8 ± 0.1, 2.0 ± 0.4, 2.8 ± 0.4, 3.8 ± 0.4, 4.6 ± 0.4, 4.9 ± 0.3, 5.4 ± 0.4, 5.2 ± 0.6, and 5.0 ± 1.0, respectively. A significant decrease in feeling score was observed at intrabag pressures of MDP + 2 mmHg (P = 0.028) and + 4 mmHg (P = 0.013), respectively, after omeprazole. No significant score changes were observed at pressures ⩾ MDP + 6 mmHg.

CONCLUSION: Although the precise mechanisms are undetermined, the present study demonstrated that omeprazole decreases mechanosensitivity to mild gastric distension.

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Key words: Functional dyspepsia; Acid exposure; Omeprazole; Barostat test; Mechanosensitivity

Peer reviewer: Akihito Nagahara, Associate Professor, Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo Bunkyo-ku, Tokyo 113-8421, Japan

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INTRODUCTION

According to Rome Ⅲ, the classification for functional gastrointestinal disorders published in 2006, functional dyspepsia (FD) is defined as the presence of one or more dyspepsia symptoms (postprandial fullness, early satiation, epigastric pain, or epigastric burning) that originate from the gastroduodenal region in the absence of any organic, systemic, or metabolic disease that is likely to explain the symptoms[1]. Alteration of gastric motility, visceral hypersensitivity, impaired accommodation of meals, gastritis induced by *Helicobacter pylori* (*H. pylori*) infection, and dysfunction of the central nervous system have all been implicated in the pathophysiology of FD[2-4]. Duodenal acidification increases proximal gastric mechanosensitivity, induces proximal gastric relaxation, and seems to inhibit the proximal gastric accommodation to a meal[5]. Hypersensitivity not only to acid in the duodenum but also gastric distension has been proposed among the possible mechanisms of FD. Thus, the mechanism by which decreased duodenal acid exposure affects symptoms of dyspepsia warrants investigation.

Omeprazole, a proton pump inhibitor that strongly decreases acid secretion, has been prescribed in the treatment of FD worldwide. This is the best first-line treatment when compared with ranitidine, cisapride, and placebo for primary care of *H. pylori*-negative dyspepsia patients[6,7]. However, changes in patients' perceptions of gastric distention after omeprazole administration have yet to be clarified. The gastric barostat test has been reported to be the gold standard method for evaluating gastric perception[8-11]. In the present study, we examined the effects of omeprazole on mechanosensitivity in humans using the barostat test.

MATERIALS AND METHODS

Study subjects

Ten healthy volunteers were recruited (8 men, 2 women; mean age, 31.6 ± 2.1 years; range, 23 to 41 years). None of the subjects had any history of gastrointestinal disease, nor were they taking any medications. Per individual interviews, no subjects had any dyspepsia symptoms (postprandial fullness, early satiation, epigastric pain, or epigastric burning). A check for *H. pylori* infection was not requested. Written informed consent was obtained from each participant. The ethics committee of Aichi Medical University approved all the study protocols (No. 369-3).

Gastric barostat

The barostat system was set up as reported previously[12]. After the patient had fasted for 12 h, a double lumen polyvinyl tube with an adherent finely folded plastic bag (Mui Scientific, Mississauga, Ontario, Canada) was introduced through the mouth into the stomach under fluorography. The polyvinyl tube was then connected to a barostat device (Distender series Ⅱ TM; G and J Electronics, North York, Ontario, Canada). The bag was unfolded by inflation with 300 mL of air and was positioned in the proximal stomach with the subject in a recumbent position, after which the bag was deflated. Subjects were then positioned so they were sitting comfortably with their knees bent (80°) and trunk upright.

Gastric sensitivity to intragastric bag distension

After a 30-min adaptation period, minimal distending pressure (MDP) was determined by increasing the intrabag pressure by 1 mmHg every 3 min until a volume of 30 mL was reached. Gastric perception of distention by a barostat device was examined[12]. Isobaric distensions were performed in stepwise increments of 2 mmHg (2 min each) starting from the MDP while the corresponding intragastric volume was recorded continuously. Subjects were instructed to score feelings in the upper abdomen at the end of every distending step using a graphic rating scale that included verbal descriptors, as follows: 0, no perception; 1, weak/vague; 2, weak but significant; 3, moderate/vague; 4, moderate but significant; 5, severe discomfort; and 6, unbearable pain. The endpoint of each sequence of distensions was established at an intrabag volume of 750 mL or when the subject reported unbearable pain (score 6).

Effects of omeprazole on the barostat test

This study was investigated the effects of omeprazole (Omepral®; AstraZenca, Osaka, Japan) on the barostat test. After the first barostat test, 10 volunteers received omeprazole (20 mg, once after dinner) as the medication for 1 wk. The second barostat study was performed on the morning after the last medication.

Statistical analysis

Data are expressed as mean ± SE. Statistical evaluation was performed using one way-analysis of variance. Values of $P < 0.05$ were considered statistically significant. Analyses were performed using a personal computer with Office Excel 2003 software (Microsoft, Redmond, WA, United States), and Analyse-it Software (Analyse-it Software, Ltd., Road Leeds, United Kingdom).

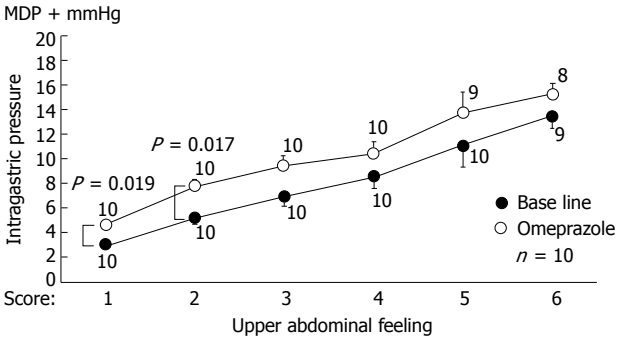


Figure 1 Intragastric pressure by a barostat device at each score of upper abdominal feeling. The distention pressure required to induce a weak/vague feeling (score 1) in the upper abdomen increased from 3.0 ± 0.5 mmHg over the minimal distending pressure (MDP) at baseline to 4.6 ± 0.4 mmHg over the MDP after omeprazole, $P = 0.019$. The distention pressure to induce a weak but significant feeling (score 2) increased from 5.2 ± 0.7 mmHg to 7.8 ± 0.6 mmHg, $P = 0.017$. The *n* sizes for each data point are indicated on the graph.

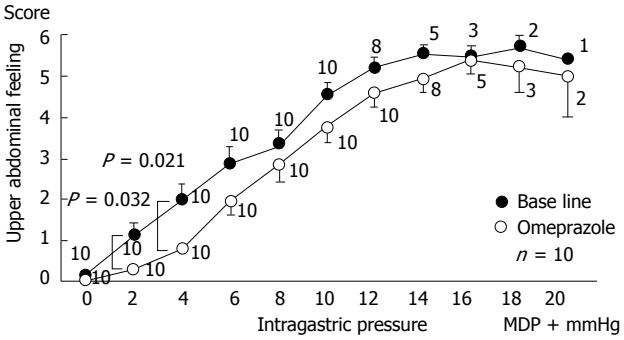


Figure 2 Score of upper abdominal feeling during gastric distension by a barostat device. The mean scores at the intrabag pressures of minimal distending pressure (MDP) + 2 mmHg and + 4 mmHg were decreased from baseline after omeprazole: 1.1 ± 0.3 *vs* 0.3 ± 0.1, $P = 0.028$; and 2.0 ± 0.4 *vs* 0.8 ± 0.1, $P = 0.0132$, respectively. No significant changes were observed at pressures > MDP + 4 mmHg. The *n* sizes for each data point are indicated on the graph.

RESULTS

No adverse effects were observed with omeprazole. Oral intubation with subsequent positioning of the barostat bag was well-tolerated by all subjects. The mean MDP before and after omeprazole was 6.3 ± 0.3 mmHg and 6.2 ± 0.5 mmHg, respectively. One subject at baseline and 2 after omeprazole did not reach discomfort scores of 6 at the maximum bag volume of 750 mL.

The distention pressures (mmHg) required to induce scores of weak/vague (score 1, $n = 10$), weak but significant (score 2, $n = 10$), moderate/vague (score 3, $n = 10$), moderate but significant (score 4, $n = 10$), severe discomfort (score 5, $n = 10$), and unbearable pain (score 6, $n = 9$) were 3.0 ± 0.5, 5.2 ± 0.7, 7.0 ± 1.0, 8.6 ± 0.9, 11.1 ± 0.8, and 13.5 ± 0.9, respectively, at baseline. After 1 week of daily omeprazole, the distension pressure required to induce the same scores ($n = 10$ for scores 1 to 4; $n = 9$, score 5; $n = 8$, score 6) were 4.6 ± 0.4, 7.8 ± 0.6, 9.4 ± 0.9, 10.4 ± 1.0, 13.8 ± 1.8, and 15.3 ± 1.0, respectively. The distended pressure needed to reach scores of 1 ($P = 0.019$) and 2 ($P = 0.017$) (Figure 1) increased significantly after omeprazole. No changes in pressure were demonstrated for other scores after treatment.

Eight subjects scored 0 for the MDP at baseline and 9 scored 0 for the MDP after omeprazole. The mean discomfort scores (± SE) at MDP before and after omeprazole were 0.13 ± 0.09 and 0.04 ± 0.04, respectively.

The mean scores induced by each MDP + 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 mmHg were 1.1 ± 0.3 ($n = 10$), 2.0 ± 0.4 ($n = 10$), 2.9 ± 0.5 ($n = 10$), 3.3 ± 0.4 ($n = 10$), 4.6 ± 0.3 ($n = 10$), 5.2 ± 0.3 ($n = 8$), 5.5 ± 0.2 ($n = 5$), 5.5 ± 0.3 ($n = 3$), 5.7 ± 0.3 ($n = 2$), and 5.4 ($n = 1$), respectively. After omeprazole, the abdominal feeling scores for the same incremental pressures over MDP were 0.3 ± 0.1 ($n = 10$), 0.8 ± 0.1 ($n = 10$), 2.0 ± 0.4 ($n = 10$), 2.8 ± 0.4 ($n = 10$), 3.8 ± 0.4 ($n = 10$), 4.6 ± 0.4 ($n = 10$), 4.9 ± 0.3 ($n = 8$), 5.4 ± 0.4 ($n = 5$), 5.2 ± 0.6 ($n = 3$), and 5.0 ± 1.0 ($n = 2$), respectively. A significant decrease in the feeling score was observed at intrabag pressures of MDP + 2 mmHg ($P = 0.028$) and + 4 mmHg ($P = 0.013$), respectively, after omeprazole (Figure 2). No significant score changes were seen at pressures ⩾ MDP + 6 mmHg.

DISCUSSION

In this study, we have shown for the first time the inhibitory effect of 1 wk of treatment with omeprazole on perception of mechanical distension by a barostat device. Namely, a significant increase was observed in the pressure required to induce a weak/vague feeling (score 1) and a weak but significant feeling (score 2). In other words, the perceived intensity of MDP + 2 and + 4 mmHg were significantly decreased after omeprazole treatment.

We recruited normal volunteers who were paid for their participation, and not all participants scored a weak/vague abdominal feeling (score 1) at intrabag pressure of MDP, indicating that the results obtained in the present study are limited to dyspepsia-free normal subjects.

The mechanisms by which omeprazole decreases perceptions of discomfort associated with gastric distension remain unknown. Omeprazole is a proton pump inhibitor that strongly decreases acid secretion. In a previous report of 5-d treatment with omeprazole at a similar dose (20 mg) to the present study, the number of hours with intragastric pH greater than 4 during a 24-h period was found to be 10.5 h[13]. Omeprazole has been reported to be most effective in patients who rated epigastric pain or heartburn as their most bothersome symptom[6,7]. Visceral hypersensitivity as well as gastric dysmotility, impaired accommodation to the meals, *H. pylori*-induced gastritis, and dysfunction of the central nervous system have all been implicated in the pathophysiology of FD[2-4]. Hypersensitivity to gastric distension and acid has especially been demonstrated in patients with FD. After the first report that gastric distension-induced perception was significantly increased in subjects with FD compared with controls in 1991[14], it has been reported that the hypersensitivity against distension might present in 34% to 66% of patients with FD[15]. In addition, duodenal acidification-

induced dyspeptic symptoms occur more significantly in patients with FD than in healthy volunteers[16].

A growing set of evidence has clarified the role of acid on dyspeptic symptoms and mechanosensitivity. First, acid infusion into the stomach induced dysmotility-like predominant dyspeptic symptoms in healthy Japanese control subjects[17]. Second, intraluminal infusion of hydrochloric acid affected sensitization of stomach mucosal mechanoreceptors[18]. Next, distal esophageal infusion also increased gastric sensitivity to distension[19]. Finally, intraduodenal acid infusion increased fundic compliance, decreased the tone and phasic contractile activities of the fundus, and increased proximal gastric mechanosensitivity[5,20,21]. These data suggest that the presence of acid in the upper gut might enhance mechanosensitivity. This putative mechanism has been supported by animal studies. Protons evoke multiple currents in primary afferent neurons that are carried by several acid-sensitive ion channels. Among these, acid-sensing ion channels (ASICs) and transient receptor potential vanilloid-1 (TRPV1) ion channels have been most thoroughly studied[22-24]. Taken together, it is speculated that an omeprazole-induced decrease in acid secretion followed by the downregulation of chemoreceptors might suppress distension-induced mechanosensitivity, resulting in the reduced perception of gastric distension demonstrated in the present study.

In this study, the inhibitory effect of omeprazole on perception induced by intragastric bag distension was demonstrated only with mild distension stimulus and weak feeling in the abdomen in normal volunteers. The precise reasons for these limited effects are unclear. Jones *et al*[22] demonstrated that both TRPV1 and ASIC3 knock-out mice were significantly less sensitive to colon distension, with an average response magnitude only 58% and 50% of controls, respectively. The data may suggest that afferent nociceptive signal transfer occurs mainly *via* a mechanoreceptor, rather than a chemoreceptor at moderate and severe distension.

It is well-known that gut infection is one of the risk factors for functional gastrointestinal disorders such as irritable bowel syndrome and FD[25]. Visceral hypersensitivity after infection has been reported in both animals[26] and humans[27]. Proton pump inhibitors have been reported to have potential anti-inflammatory effects apart from acid suppression[28]. Therefore, it is possible that the omeprazole-induced decreased perception of gastric distension might be mediated by the anti-inflammatory action of omeprazole.

In conclusion, although the precise mechanisms are undetermined, the present study demonstrated that omeprazole decreases mechanosensitivity to mild gastric distension, suggesting that the drug might be effective in the treatment of FD.

COMMENTS

Background

Hypersensitivity to gastric distension has been proposed among the possible mechanisms of functional dyspepsia (FD). Omeprazole has been prescribed in the treatment of FD. They examined the effects of omeprazole on mechanosensitivity in humans.

Research frontiers

The gastric barostat test is the gold standard method for evaluating gastric perception. They examined the effects of omeprazole on mechanosensitivity in humans using the barostat test.

Innovations and breakthroughs

Omeprazole is a proton pump inhibitor that strongly decreases acid secretion. The data suggest that suppression of acid secretion may decrease mechanosensitivity of the proximal stomach as well as dyspeptic symptoms.

Applications

Although the precise mechanisms are undetermined, the present study demonstrated that omeprazole decreases mechanosensitivity to mild gastric distension.

Terminology

These studies used the gastric barostat test in which a plastic bag with a double lumen polyvinyl tube was introduced into the stomach under fluorography. The tube was connected to a barostat device, which is a computer-controlled pump. Isobaric distensions were performed in stepwise increments. This method is the gold standard for evaluating gastric perception.

Peer review

This is a good descriptive study in which authors evaluate the effects of omeprazole on gastric mechanosensitivity in humans. The results are interesting and suggest that omeprazole decreases mechanosensitivity to mild gastric distension.

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BRIEF ARTICLE

Thioredoxin and thioredoxin-interacting protein as prognostic markers for gastric cancer recurrence

Jae Yun Lim, Sun Och Yoon, Soon Won Hong, Jong Won Kim, Seung Ho Choi, Jae Yong Cho

Jae Yun Lim, Jae Yong Cho, Department of Medical Oncology, Gangnam Severance Cancer Hospital, Yonsei University College of Medicine, Seoul 135-720, South Korea
Sun Och Yoon, Soon Won Hong, Department of Pathology, Gangnam Severance Cancer Hospital, Yonsei University College of Medicine, Seoul 135-720, South Korea
Jong Won Kim, Seung Ho Choi, Department of Surgery, Gangnam Severance Cancer Hospital, Yonsei University College of Medicine, Seoul 135-720, South Korea
Author contributions: Lim JY and Cho JY designed the study; Lim JY, Yoon SO, Hong SW, Kim JW and Choi SH contributed to performing experiment and acquisition of data; Lim JY, Yoon SO and Cho JY analyzed and interpreted the data; Lim JY and Cho JY wrote the paper.
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Correspondence to: Dr. Jae Yong Cho, Department of Medical Oncology, Gangnam Severance Cancer Hospital, Yonsei University College of Medicine, 712 Eonjuro, Gangnam-gu, Seoul 135-720, South Korea. chojy@yuhs.ac
Telephone: +82-2-20194363 Fax: +82-2-34633882
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Abstract

AIM: To evaluate the potential of thioredoxin (TXN) and thioredoxin-interacting protein (TXNIP) expression as biomarkers for predicting gastric cancer recurrence.

METHODS: TXN and TXNIP expression levels were acquired from gene expression microarray data for 65 human gastric cancer tissues. We determined whether each gene expression level was associated with cancer recurrence and investigated the relationship between the two genes. For validation, the expression levels of TXN and TXNIP were measured by quantitative real-time reverse transcription polymerase chain reaction in 68 independent stage Ⅲ gastric cancer patients. The correlation between gene expression and cancer prognosis was evaluated. Immunohistochemical staining was performed to investigate the protein expression levels of TXN and TXNIP and to characterize the expression patterns of each protein.

RESULTS: TXN was a prognosis-related gene (P = 0.009), whereas TXNIP, a TXN inhibitor, demonstrated a negative correlation with TXN in the gene expression microarray data. In the 68 stage Ⅲ patients, the expression levels of both TXN and TXNIP had a statistically significant effect on recurrence-free survival (RFS, P = 0.008 and P = 0.036, respectively). The low TXN and high TXNIP expression group exhibited a better prognosis than the other groups, and the high TXN and low TXNIP expression group exhibited a poorer prognosis (P < 0.001 for RFS and P = 0.001 for overall survival). More than half of the patients in the simultaneously high TXN and low TXNIP expression group experienced a recurrence within 1 year after curative surgery, and the 5-year survival rate of the patients in this group was 29%, compared with 89% in the low TXN and high TXNIP expression group. The TXN protein was overexpressed in 65% of the gastric cancer tissues, whereas the TXNIP protein was underexpressed in 85% of the cancer cells. In a correlation analysis, TXN and TXNIP were highly correlated with many oncogenes and tumor suppressors as well as with genes related to energy, protein synthesis and autophagy.

CONCLUSION: TXN and TXNIP are promising prognostic markers for gastric cancer, and performing personalized adjuvant treatment based on TXN and TXNIP expression levels would be an effective practice in the treatment of gastric cancer.

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Key words: Gastric cancer; Thioredoxin; Thioredoxin-interacting protein; Biomarker; Prognosis

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Gastroenterology, Gachon Graduate School of Medicine, 7-45 Songdo-dong, Yeonsu-gu, Incheon 406-840, South Korea; Hitoshi Tsuda, MD, PhD, Diagnostic Pathology Section, Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

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INTRODUCTION

Gastric cancer is the second leading cause of global cancer mortality, and it has the highest mortality rates in East Asia, including South Korea, Japan and China[1]. The standard treatment for gastric cancer is surgery. However, relapses occur in many patients who undergo curative resection, even after adjuvant therapy[2,3]. Even among individuals with the same stage of cancer, gastric cancer patients present with diverse clinical manifestations and prognoses. The clinical diversity of gastric cancer arises from its molecular biological diversity, which is caused by changes in different genes. Molecular markers are important in predicting patient outcome and in personalizing treatments according to the individual biology of the patient. Importantly, in the adjuvant treatment setting, uniform adjuvant treatment after curative resection of gastric cancer has been performed regardless of the individual molecular prognostic markers of the cancer. However, cancer researchers have identified biomarkers that can predict recurrence and survival[4-8], and doctors should consider using biomarker-driven personalized adjuvant treatment.

Thioredoxin (TXN) is a low-molecular-weight redox protein and a putative oncoprotein that provides growth and survival advantages to tumor cells through the activation of redox-sensitive transcription factors, such as nuclear factor kappa B (NF-κB), p53, and activator protein-1 (AP-1)[9,10]. TXN inhibits apoptosis via apoptosis signaling kinase-1 (ASK-1) and phosphatase and tensin homolog (PTEN)[11]. TXN also induces hypoxia-inducible factor-1α (HIF-1α), which increases the production of vascular endothelial growth factor and leads to tumor angiogenesis and drug resistance[12]. The overexpression of TXN has been found in several cancers, including lung[13], pancreatic[14], cervical[15], and colorectal cancers[16] as well as hepatomas[17]. The increased expression of TXN in tumors has been associated with decreased patient survival in several cancers and with resistance to anticancer drugs[18,19]. The TXN-interacting protein (TXNIP), also known as vitamin D3 up-regulated protein-1 (VDUP1) and TXN-binding protein-2 (TBP-2), inhibits the interaction between TXN and other factors. TXNIP overexpression inhibits TXN activity, which in turn inhibits tumor cell proliferation and cell cycle progression[20,21]. Recently, it was reported that knockout of the *TXNIP* gene in a mouse model induced *Helicobacter pylori* (*H. pylori*)-related gastric cancer[22]. However, there is little information regarding the prognostic value of the expression level of TXN and TXNIP in gastric cancer.

In this study, we evaluated the use of TXN expression combined with TXNIP expression as prognostic markers to individualize the postoperative treatment strategy following gastric cancer removal.

MATERIALS AND METHODS

Gene expression microarray data analysis

The previously generated gene expression data from gastric cancer patients are available in the NCBI's GEO public database (microarray data accession number, GSE13861)[23]. Sixty-five gastric cancer patients underwent curative surgery as a primary treatment, with clinical data obtained from the Yonsei University Severance Hospital (Table 1). Sixty-five surgically removed frozen gastric adenocarcinoma tissues and 19 normal surrounding tissue samples were used for the microarray experiments. The total RNA was extracted from the fresh-frozen tissues using a mirVana RNA Isolation Labeling Kit (Ambion, Austin, TX, United States). For the labeling and hybridization, 500 ng of total RNA was used, according to the manufacturer's protocols (Human-HT12 v.3 Expression BeadChip, Illumina, San Diego, CA, United States). The microarray data were normalized using the quantile normalization method in the Linear Models for Microarray Data package in the R language environment. The expression level of each gene was transformed into a log2 base prior to further analysis. The random variance *t* test was applied to identify the differentially expressed genes between the two tissue types. The gene expression differences were considered significant if the *P* value was less than 0.001. Cluster analysis was performed with Cluster 3.0 and TreeView[24]. Univariate analysis was performed by dividing the patients into two groups based on the median value of each gene expression level to search for prognostic genes.

Quantitative reverse transcription-polymerase chain reaction and analysis

Paraffin-embedded cancer tissues were collected from gastric adenocarcinoma patients who underwent curative surgery between 1999 and 2007 as a primary treatment at Gangnam Severance Hospital. The clinical data of the patients were reviewed to obtain age, sex, tumor location, tumor differentiation, and stage based upon the American Joint Committee on Cancer 2002 criteria. The patients were followed up for more than 36 mo after surgery or until recurrence or death within 36 mo after surgery.

Sixty-eight stage Ⅲ gastric cancer tissues were chosen to validate the microarray data (Table 1). The total RNA was extracted according to the manufacturer's instructions (RecoverAll™ Total Nucleic Acid Isolation; Applied Biosystems, Foster City, CA, United States). The

Table 1 Clinicopathological factors of the gastric cancer patients

Characteristics	Microarray (*n* = 65)	qRT-PCR (*n* = 68)	TMA (*n* = 328)
Age			
mean (range), yr	63 (32-83)	56 (26-82)	57 (25-82)
Sex, *n* (%)			
Male/female	46 (71)/19 (29)	36 (53)/32 (47)	204 (62)/124 (38)
Follow up duration			
Mean (95% CI), mo	41.7 (41-42)	89.5 (79-100)	99.8 (97.5-102)
Histological type, *n* (%)			
Intestinal	23 (35)	14 (21)	100 (30)
Diffuse	42 (65)	54 (79)	228 (70)
TNM stage, *n* (%)			
Ⅰ	12 (18)	0	101 (31)
Ⅱ	11 (17)	0	79 (24)
Ⅲ	26 (40)	68 (100)	110 (33)
Ⅳ	16 (25)	0	38 (12)
Location, *n* (%)			
Cardia	5 (8)	8 (12)	25 (8)
Non cardia	60 (92)	60 (88)	303 (92)
Adjuvant chemotherapy, *n* (%)			
Yes	49 (75)	59 (87)	230 (70)
No	16 (25)	9 (13)	98 (30)

qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; TMA: Tissue microarray; TNM: Tumor node metastasis.

TXN and *TXNIP* genes were assayed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with TaqMan gene-specific primers (Applied Biosystems, Foster City, CA, United States). Real-time RT-PCR amplification was performed using the 7900HT Fast Real-Time PCR System with a 384-well block module (Applied Biosystems, Foster City, CA, United States). The cycling conditions were as follows: 48 ℃ for 30 min and 95 ℃ for 10 min, followed by 40 cycles at 95 ℃ for 15 s and at 60 ℃ for 60 s. The relative amounts of mRNA were calculated from the threshold cycle (C_T) number using the expression of β-2 microglobulin as an endogenous control. All of the experiments were performed in triplicate, and the values were averaged.

Tissue microarray construction and immunohistochemical staining

Paraffin-embedded tissue microarray blocks of gastric cancer tissue specimens were created from tissues from 328 patients. Each block had 3-mm cores of gastric cancer tissue. The 4-μm thick sections were deparaffinized and processed to block endogenous peroxidase activity. Next, an antigen retrieval step was performed. Subsequently, primary anti-TXN (Polyclonal, 1:500, Abcam, Cambridge, MA, United States) and anti-TXNIP antibodies (Polyclonal, 1:100 Sigma, St. Louis, MO, United States) were applied to the sections. The sections were then incubated with a secondary antibody (HRP-rabbit/mouse), and the stains were developed using a Nova-RED substrate kit (VECTOR Laboratory, Burlingame, CA, United States). The samples were then counterstained with Harris hematoxylin.

The TXN and TXNIP protein expression levels were evaluated by two pathologists. Over-expression was defined as staining higher in more than 50% of cancer cells compared to the matching normal cells, regardless of cytoplasmic or nuclear location. Underexpression was defined as no staining or staining positivity lower than that of the matching normal tissue, and normal expression was defined as a level of staining positivity similar to that of the matching normal tissue. For slides that were heterogeneously stained within a tumor, we graded the highest intensity within the tumor.

Statistical analysis

The BRB-Array Tools system was used for the analysis of the microarray data. Statistical analysis were primarily performed with PASW Statistics 17.0 (SPSS Inc., Chicago, IL, United States). A χ^2 test was used to compare the difference between the groups. Kaplan-Meier plots and a log-rank test were used to estimate patient survival. Associations between the expression levels of the two targets and in-trans correlation were analyzed using the Pearson correlation coefficient. A *P* value of less than 0.05 was considered statistically significant, and all tests were two tailed.

RESULTS

Expression of TXN mRNA in microarray data for gastric cancer

First, a hierarchical clustering analysis was applied to the gene expression data from the 65 human primary tumor tissue samples. Unsupervised clustering revealed 2 distinctive subtypes with clear differences in overall gene expression patterns[23]. Recurrence-free survival (RFS) was found to differ significantly between the 2 clusters ($P = 0.001$ by the log rank test), indicating that the molecular features of these tumors reflected in gene expression patterns might be strong independent predictors of clinical outcomes.

We next sought to identify genes whose expression was unique to the poor prognostic subgroup by cross-comparing gene lists. Between the two groups, 1258 genes were differentially expressed ($P < 0.001$) and presented more than a 1.5-fold difference between the two groups. Next, a univariate analysis identified 84 prognostic genes ($P < 0.01$). Of the 84 genes, TXN was a prognosis-related gene ($P = 0.009$ by univariate analysis) and was up-regulated in the poor prognostic group. From the correlation analysis between the expression levels of TXN and associated genes, we determined the relative correlations. TXNIP, a TXN inhibitor, demonstrated a negative correlation with TXN ($r = -0.295$, $P = 0.024$). The survival analysis based on the hierarchical clustering of TXN- or TXNIP-related genes demonstrated that each cluster influenced patient survival ($P = 0.025$ and $P = 0.036$, respectively) (Figure 1).

qRT-PCR validation of TXN and TXNIP expression

To determine if *TXN* and *TXNIP* gene expression was associated with prognosis among patients with the same

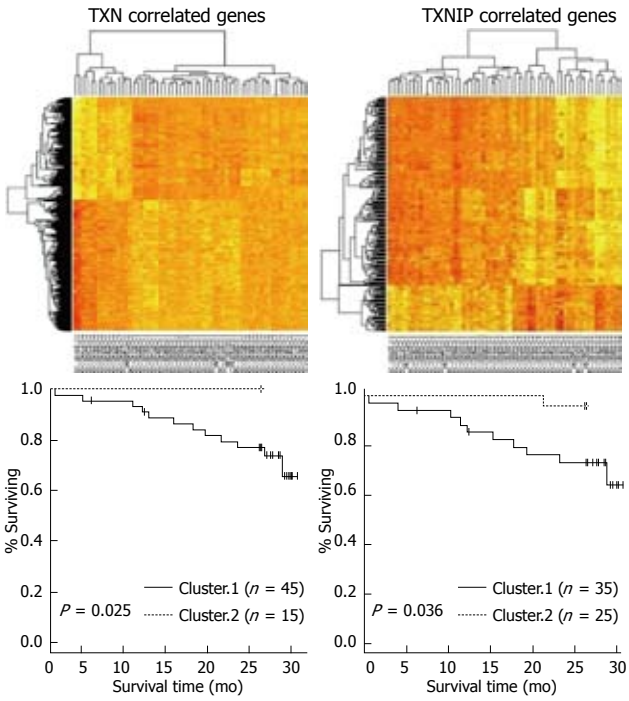


Figure 1 Survival analysis based on hierarchical clustering of thioredoxin or thioredoxin-interacting protein correlated genes.

stage of cancer, 68 stage Ⅲ patients were randomly selected for qRT-PCR analysis (Table 1). The median follow-up duration after curative resection was 89.5 mo. By the last follow-up visit, 44 patients had experienced a recurrence, and 16 patients had died of gastric cancer. The patients were divided into high or low expression groups according to the TXN and TXNIP expression levels. RFS and overall survival (OS) were analyzed between the two groups (Figure 2A-D). Both TXN and TXNIP expression influenced RFS, and these associations were significant (P = 0.008 and P = 0.036, respectively). The patients in the high TXN expression or low TXNIP expression groups presented a poor prognosis. TXN expression also influenced OS (P = 0.015), but TXNIP expression did not have a significant relationship with OS (P = 0.053).

Stage Ⅲ patients were classified into the following three combination groups based on the TXN and TXNIP expression levels: Group 1, simultaneously low TXN and high TXNIP levels (n = 20); Group 2, neither Group 1 nor Group 3 (n = 35); and Group 3, simultaneously high TXN and low TXNIP levels (n = 13). As expected, Group 1 exhibited a better prognosis than the other groups, and Group 3 presented with a poor prognosis (Figure 2E and F; P < 0.001 for RFS and P = 0.001 for OS). More than half of the Group 3 patients experienced a recurrence within 1 year after curative surgery, and their 5-year survival rate was one-third (29%) that of Group 1 (89%) (Table 2). There was no relationship between the TXN/TXNIP expression levels and either tumor stage or histological cell type.

Immunohistochemical assay of TXN and TXNIP expression

The gastric glands were relatively well stained with the anti-TXN antibody regardless of the presence of cancerous and non-cancerous lesions based on the immunohistochemical assay results. Thioredoxin was overexpressed in approximately 65% of cancer tissues regardless of the histological type and TNM stage (Table 3). Thioredoxin staining was observed in the cytoplasm or nucleus or in both areas of the cancer cells. The histological location of thioredoxin in the cytoplasm or the nucleus in cancer tissues varied among patients, and there was heterogeneity in the staining intensity within the individual samples. In contrast to the staining with the anti-thioredoxin antibody, the staining of the gastric cancer tissue with the anti-TXNIP antibody was weak. TXNIP was underexpressed in almost 85% of the cancer cells and was found in the cytoplasm or nucleus of the cancer cells. There was a tendency for TXNIP to be underexpressed in two poor prognosis groups: diffuse histology type (P = 0.009) and high-stage gastric cancer (P = 0.011) (Table 3). Representative immunohistochemical staining results are shown in Figure 3.

Table 2 Prognoses of stage Ⅲ gastric cancer patients according to the thioredoxin and thioredoxin-interacting protein gene expression levels

Group	Mean RFS, (95% CI), mo	3-yr recurrence-free rate (%)	5-yr survival rate (%)
1: Low TXN and high TXNIP	Non applicable	70	89
2: Neither group 1 nor group 3	36.7 (15.5-57.9)	51	73
3: High TXN and low TXNIP	11.9 (9.5-14.2)	7	29
P value by log rank test		P < 0.001	P = 0.001

RFS: Recurrence-free survival; TXN: Thioredoxin; TXNIP: Thioredoxin-interacting protein.

Table 3 Protein expression of thioredoxin and thioredoxin-interacting protein in gastric cancers

Characteristic (n)	TXN overexpression n (%)	TXNIP underexpression n (%)
Total (328)	213 (65)	278 (85)
Histologic type		
Diffuse type (228)	149 (65)	201 (88)
Intestinal type (100)	64 (64)	77 (77)
	P = 0.9	P = 0.012
Stage		
Stage Ⅰ / Ⅱ (180)	116 (64)	142 (79)
Stage Ⅲ / Ⅳ (148)	97 (66)	136 (92)
	P = 0.767	P = 0.009

TXN: Thioredoxin; TXNIP: Thioredoxin-interacting protein.

Bioinformatic in-trans correlation analysis of TXN and TXNIP expression

Approximately 7000 genes were significantly correlated with TXN according to the bioinformatic in-trans correlation analysis of TXN expression with whole mRNA-expressing genes (P < 0.001). These findings suggest that TXN plays an important role in gastric cancer. Glutaredoxin 2 (GLRX2) and peroxiredoxin 4 (PRDX4) were highly correlated with TXN (r = 0.76, P < 0.001 and r = 0.72, P < 0.001, respectively; Table 4), and this correlation explains the function of TXN as a reactive

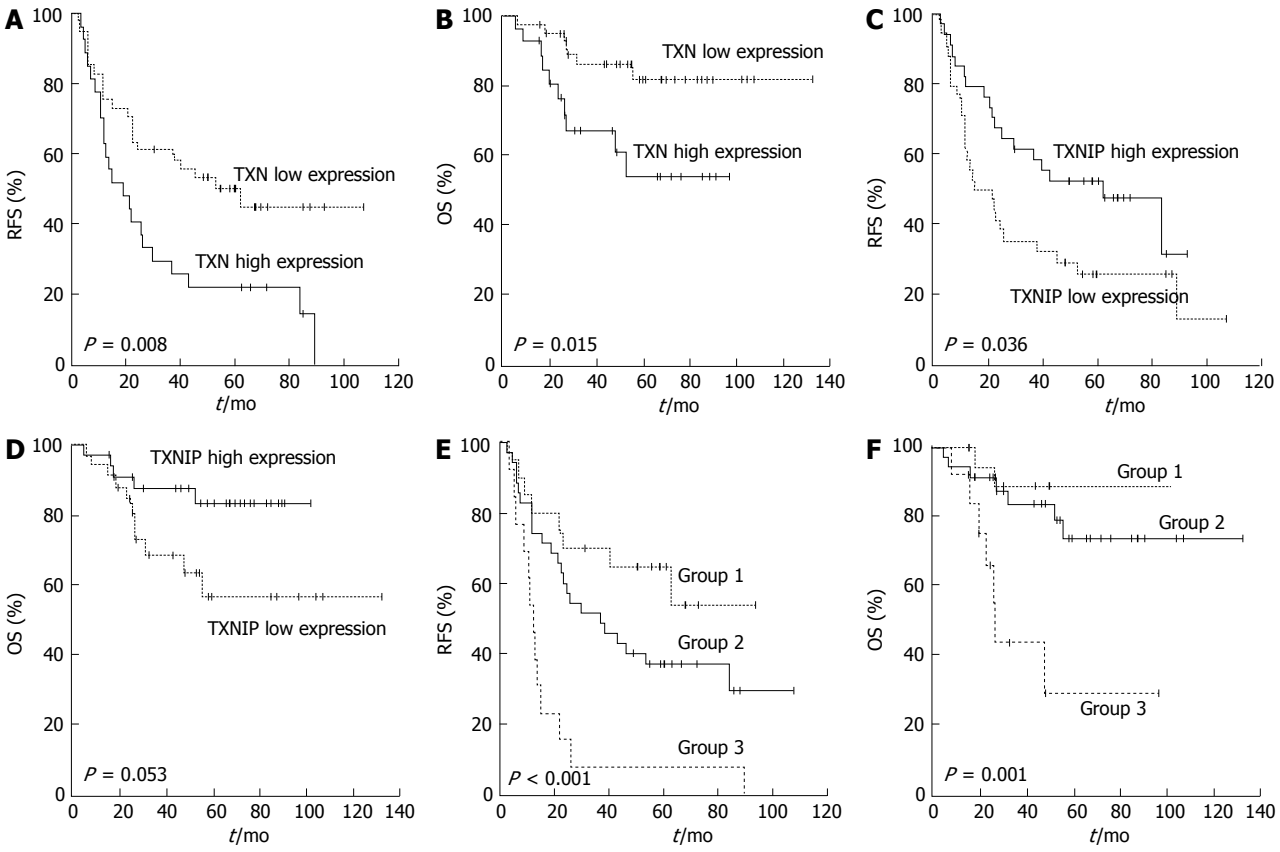


Figure 2 Kaplan-Meier plot of recurrence-free survival and overall survival according to gene expression. Gene expression was measured using quantitative reverse transcription-polymerase chain reaction. Stage III gastric cancer patients were divided into two or three groups according to their gene expression levels. A, B: Survival curves according to thioredoxin (TXN) expression level; C, D: Survival curves according to thioredoxin-interacting protein (TXNIP) expression levels; E, F: Survival curves according to the combination of the TXN and TXNIP expression levels (Group 1: Simultaneous low TXN and high TXNIP expression; Group 2: Neither group 1 nor group 3; Group 3: Simultaneous high TXN and low TXNIP expression). The prognosis was compared across the groups using the log-rank test.

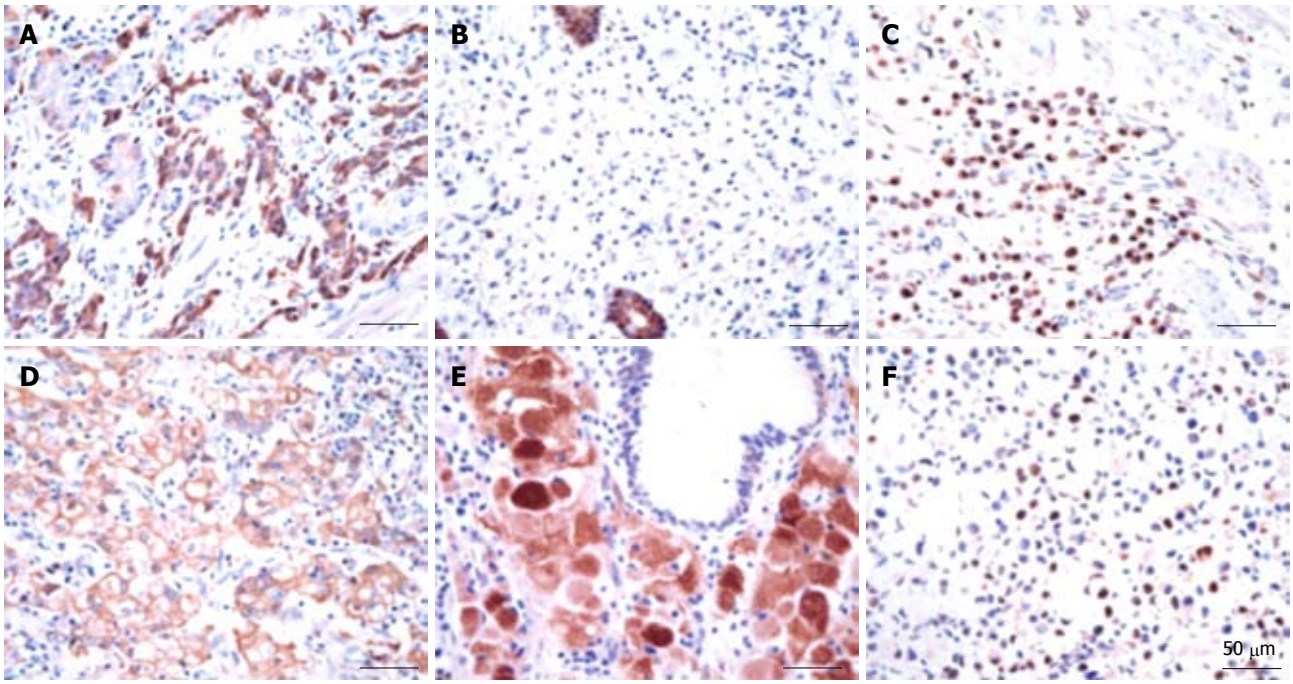


Figure 3 Representative images of immunohistochemical assay (×400). A: The cytoplasm of gastric cancer cells was strongly stained with the anti-thioredoxin (TXN) Ab; B: The cytoplasm of gastric cancer cells was not stained with the anti-TXN Ab; C: The nuclei of gastric cancer cells were strongly stained with the anti-TXN Ab; D; The nuclei of gastric cancer cells were not stained with the anti-TXN Ab; E: The cytoplasm of gastric cancer cells was strongly stained with the anti-thioredoxin-interacting protein (TXNIP) Ab, but the nuclei were not stained; F: The nuclei of gastric cancer cells were strongly stained with the anti-TXNIP Ab, but the cytoplasm was not stained.

Table 4 In-trans correlation analysis of thioredoxin and thioredoxin-interacting protein with whole mRNA expressing genes

Symbol	Gene name	Correlation with TXN		Correlation with TXNIP	
		r	*P* value	*r*	*P* value
TXN	Thioredoxin	1.000	0.000	-0.295	0.022
RPL6	Ribosomal protein L6	0.779	< 0.001	-0.335	0.009
GLRX2	Glutaredoxin 2	0.764	< 0.001	-0.314	0.015
RPL29	Ribosomal protein L29	0.757	< 0.001	-0.360	0.005
MRPL22	Mitochondrial ribosomal protein L22	0.751	< 0.001	-0.225	0.085
MRPL42	Mitochondrial ribosomal protein L42	0.734	< 0.001	-0.181	0.166
MRPS17	Mitochondrial ribosomal protein S17	0.732	< 0.001	-0.387	0.002
ATP5S	ATP synthase-coupling factor B	0.728	< 0.001	-0.182	0.165
PRDX4	Peroxiredoxin 4	0.722	< 0.001	-0.321	0.012
AURKA	Aurora kinase A	0.611	< 0.001	-0.486	< 0.001
ERO1L	ERO1-like	0.586	< 0.001	-0.200	0.125
HIG2	Chromosome 7 open reading frame 68	0.574	< 0.001	-0.089	0.498
CCNB1	Cyclin B1	0.563	< 0.001	-0.503	< 0.001
ERBB3	HER3	0.004	0.975	-0.536	< 0.001
TXNIP	Thioredoxin interacting protein	-0.295	0.022	1.000	0.000
ATG12	ATG12 autophagy related 12 homolog	-0.482	< 0.001	0.072	0.582
ATG16L2	ATG16 autophagy related 16-like 2	-0.722	< 0.001	0.216	0.098
ATG10	Autophagy related 10 homolog	-0.756	< 0.001	0.266	0.040

TXN: Thioredoxin; TXNIP: Thioredoxin-interacting protein.

oxygen species (ROS) scavenger with these known redox molecules. Ribosomal proteins and mitochondrial ribosomal proteins (i.e., RPL6, RPL29, MRPL22, MRPL42, MRPS17 and ATP5S) were highly correlated with TXN ($P < 0.001$; Table 4), which suggests the active involvement of TXN in protein synthesis and mitochondrial ATP synthesis for energy production. TXNIP was negatively correlated with TXN and known poor prognostic markers, such as AURKA, ERBB3, CCNB1, and many genes that are significantly correlated with TXN (Table 4). These results indirectly support the functional role of the TXNIP in the inhibition of TXN.

DISCUSSION

We demonstrated that TXN and TXNIP are poor and good prognostic gastric cancer markers, respectively. In stage Ⅲ patients who underwent curative gastrectomy, the 3-year recurrence-free rate ($P < 0.001$) and the 5-year survival rate ($P = 0.001$) were significantly different between patients with different TXN and TXNIP expression levels (Table 2). High TXN and low TXNIP patients had a definitively poor prognosis. Most of these patients experienced a recurrence within 2 years and died within 4 years. Thus, it is necessary to perform intensive treatment and plan post-adjuvant treatment for these patients.

The gene expression microarray data imply that TXN plays an important role in gastric cancer. TXN functions as a ROS scavenger with known redox molecules, such as GLRX2 and PRDX4, and TXN is actively involved in protein synthesis and mitochondrial ATP synthesis for energy production. Hypoxia-induced genes [i.e., ERO1L and hypoxia inducible gene 2 (*HIG2*)] were significantly correlated with TXN. Tumors with high TXN expression also exhibited elevated *ERO1L* and *HIG2* levels; therefore, these tumors are in a relatively high hypoxic state compared with tumors with low TXN expression. Additionally, autophagy-related genes (i.e., *ATG10*, *ATG16L2*, and *ATG12*) were negatively correlated with TXN expression, which indirectly suggests that TXN is involved in autophagic inhibition in a hypoxic cancer state. Autophagy is associated with cancer pathogenesis and chemotherapy resistance; therefore, the function of TXN in autophagy should be elucidated through additional studies.

Grogan *et al*[25] demonstrated that TXN was localized to tumor cells and was overexpressed in gastric cancer tissues compared with the levels in normal gastric mucosa. TXN overexpression was typically found in both the nucleus and the cytoplasm of neoplastic cells. These findings are consistent with our immunohistochemical staining results. Furthermore, high TXN expression was observed regardless of the gastric cancer stage or cell type.

Our study confirmed that TXNIP was significantly underexpressed in gastric cancer tissues compared with normal tissues; furthermore, it was expressed at the lowest levels in cancer patients with poor prognoses. TXNIP is a known potent tumor suppressor whose expression is markedly decreased in various human cancers, including gastric cancer. Knockout of the TXNIP gene in a mouse model was associated with *H. pylori*-related gastric cancer[22]. In an *in vitro* experiment, TXNIP overexpression in pancreatic cells resulted in a higher basal level of apoptosis and an increased sensitivity to cisplatin and oxaliplatin[26]. In microarray data analysis, TXNIP expression was negatively correlated with the expression of TXN expression, known as poor prognostic cancer biomarkers[27-29], and many genes that are significantly correlated with TXN. These results indirectly confirm the functional role of the TXNIP in TXN inhibition. Additionally, autophagy-related genes were correlated with TXNIP, and these data support that TXNIP induces cancer cell autophagy.

In this study, we analyzed the gene expression profile

of human gastric cancer to identify potential biomarkers that could be used to classify patients according to prognosis after curative resection. We found that TXN and TXNIP were significantly associated with prognosis. TXN up-regulation and the simultaneous down-regulation of TXNIP were associated with a poor prognosis in gastric cancer patients. Bioinformatic analysis revealed that TXN and TXNIP were highly correlated with many oncogenes and tumor suppressor genes and demonstrated that TXN and TXNIP were associated with genes related to energy, protein synthesis and the modulation of autophagy under hypoxic or other stressful conditions. One of the limitations of this study is that we did not elucidate how TXN and TXNIP affect the recurrence of gastric cancer after curative resection. Further investigation of TXN and TXNIP in gastric cancer would likely identify unknown pathogenic mechanisms. In addition, because our results were derived from retrospective assessment, it is necessary to utilize *TXN* and *TXNIP* gene expression-based prediction in a prospective randomized trial(s) to validate the true clinical relevance of TXN and TXNIP. It will be beneficial to identify effective anti-tumor treatments other than the current standard adjuvant chemotherapy for gastric cancer patients with potent recurrence factor, high TXN and low TXNIP expression. Furthermore, TXN-targeted agents or those that up-modulate TXNIP could be used in targeted therapy in the treatment of gastric cancer patients who are selected based on biomarker gene signatures.

In conclusion, TXN and TXNIP are promising prognostic markers for gastric cancer, and performing personalized adjuvant treatment based on TXN and TXNIP expression levels would be an effective practice in the treatment of gastric cancer.

COMMENTS

Background

The standard of treatment for gastric cancer is surgery. After curative resection, even among individuals with the same stage of cancer, diverse recurrence patterns are present. However, uniform adjuvant treatment after curative resection has been performed. Authors evaluated the use of thioredoxin (TXN) expression combined with thioredoxin-interacting protein (TXNIP) expression as prognostic markers to individualize the postoperative treatment strategy following gastric cancer removal.

Research frontiers

TXN is putative oncoprotein that provides growth and survival advantages to tumor cells through the activation of redox-sensitive transcription factors. TXNIP is a potent tumor suppressor and inhibits the interaction between TXN and other factors. They have been recognized as cancer-related markers in diverse malignancies.

Innovations and breakthroughs

High TXN and low TXNIP expression in human gastric cancer tissue definitively related with poor prognosis of gastric cancer patients who had undergone curative resection.

Applications

The establishment of adjuvant treatment strategy based on our discovery would be useful to accomplish better outcomes after surgical treatment. The patients with high TXN and low TXNIP expressions in cancer tissue require novel treatment and should be more carefully monitored after surgery.

Terminology

Redox (reduction-oxidation) reactions: Many important biological processes involve redox reactions. Free radicals are a part of redox molecules and can become harmful to the human body if they do not reattach to the redox molecule or an antioxidant. Unsatisfied free radicals can trigger the mutation of cells they encounter and are thus cancer causing.

Peer review

This study investigated the potential of TXN and TXNIP genes as a prognostic marker after curative resection of gastric cancer. Patients with high TXN and low TXNIP expression in cancer tissue manifested significantly more recurrence and shorter survival. However, it is necessary to validate through the prospective trial and to elucidate the functional mechanism of TXN and TXNIP.

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BRIEF ARTICLE

Photodynamic therapy prolongs metal stent patency in patients with unresectable hilar cholangiocarcinoma

Tae Yoon Lee, Young Koog Cheon, Chan Sup Shim, Young Deok Cho

Tae Yoon Lee, Young Koog Cheon, Chan Sup Shim, Digestive Disease Center, Department of Internal Medicine, Konkuk University Medical Center, Konkuk University School of Medicine, Seoul 143-729, South Korea
Young Deok Cho, Institute for Digestive Research and Digestive Disease Center, Soonchunhyang University College of Medicine, Seoul 140-743, South Korea
Author contributions: Cheon YK and Shim CS designed the research; Cheon YK and Cho YD performed the research; Lee TY and Cheon YK analyzed the data; and Lee TY and Cheon YK wrote the paper.
Correspondence to: Young Koog Cheon, MD, PhD, Professor, Digestive Disease Center, Department of Internal Medicine, Konkuk University Medical Center, Konkuk University School of Medicine, 120-1 Neungdong-ro, Gwangjin-gu, Seoul 143-729, South Korea. yksky001@hanmail.net
Telephone: +82-2-20308195 Fax: +82-2-20305029
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Abstract

AIM: To evaluate the effect of photodynamic therapy (PDT) on metal stent patency in patients with unresectable hilar cholangiocarcinoma (CC).

METHODS: This was a retrospective analysis of patients with hilar CC referred to our institution from December, 1999 to January, 2011. Out of 232 patients, thirty-three patients with unresectable hilar CC were treated. Eighteen patients in the PDT group were treated with uncovered metal stents after one session of PDT. Fifteen patients in the control group were treated with metal stents alone. Porfimer sodium (2 mg/kg) was administered intravenously to PDT patients. Forty-eight hours later, PDT was administered using a diffusing fiber that was advanced across the tumor by either endoscopic retrograde cholangiopancreatography or percutaneous cholangiography. After performance of PDT, uncovered metal stents were inserted to ensure adequate decompression and bile drainage. Patient survival rates and cumulative stent patency were calculated using Kaplan-Meier analysis with the log-rank test.

RESULTS: The PDT and control patients were comparable with respect to age, gender, health status, pretreatment bilirubin, and hilar CC stage. When compared to control, the PDT group was associated with significantly prolonged stent patency (median 244 ± 66 and 177 ± 45 d, respectively, $P = 0.002$) and longer patient survival (median 356 ± 213 and 230 ± 73 d, respectively, $P = 0.006$). Early complication rates were similar between the groups (PDT group 17%, control group 13%) and all patients were treated conservatively. Stent malfunctions occurred in 14 PDT patients (78%) and 12 control patients (80%). Of these 26 patients, twenty-two were treated endoscopically and four were treated with external drainage.

CONCLUSION: Metal stenting after one session of PDT may be safe with acceptable complication rates. The PDT group was associated with a significantly longer stent patency than the control group in patients with unresectable hilar CC.

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Key words: Bile duct cancer; Palliative endoscopic stenting; Photodynamic therapy; Outcome

Peer reviewer: Jong H Moon, MD, PhD, Professor of Medicine, Digestive Disease Center, Soon Chun Hyang University Bucheon Hospital, No. 1174 Jung-Dong, Wonmi-Ku, Bucheon 420-767, South Korea

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INTRODUCTION

Hilar cholangiocarcinoma (CC) is a rare tumor that is asymptomatic early on, making it difficult to diagnose, and is associated with a high mortality[1]. Surgical resection with negative histologic margins is the most robust predictor of long-term survival. However, due to the lack of characteristic early symptoms, a definitive diagnosis is often not reached until the tumor is at an advanced stage. As a result, a large proportion of patients are beyond the scope of curative treatment by the time of diagnosis, and only palliative management is possible[2]. Palliative therapies for unresectable CC such as stent, radiotherapy and chemotherapy, have generally been disappointing in prolonging life[3]. Endoscopic or percutaneous biliary drainage alleviates jaundice, but there is no evidence that it prolongs survival.

Although endoscopic biliary metal stenting is the mainstay of palliative treatment in patients with unresectable hilar CC[4], tumor ingrowth or overgrowth is a significant problem in uncovered stents. In particular, the duration of metal stent patency for hilar CCs was shorter than that for distal bile duct cancer because the malignant hilar stricture provides an acute angle that hinders full expansion of the metal stent and promotes biliary sludge formation within. Several clinical trials have reported the therapeutic effect of photodynamic therapy (PDT) for unresectable hilar CC[5,6]. The ability of PDT to destroy cancer and neovascular cells may prolong biliary stent patency. However, the effect of PDT on stent patency has not yet been determined. Therefore, the primary aim of this study was to explore the effect of PDT on stent patency in unresectable hilar CC. We also evaluated overall survival and procedure-related complications after PDT compared with endoscopic biliary drainage alone for unresectable hilar CCs.

MATERIALS AND METHODS

Patients

This was a non-randomized, retrospective study. From December, 1999 to January, 2011, 232 patients with hilar cholangiocarcinoma were referred to our institution. This study included patients with unresectable hilar CC without chemoradiation who were palliated with only metal stents (control group) or with one session of PDT followed by metal stents (PDT group). Patients were deemed inoperable if they met the following criteria: the presence of type Bismuth-Corlette Ⅳ lesions, advanced type Ⅲ lesions containing T3 tumors, or other types Ⅱ-Ⅲ tumors if surgery was contraindicated due to lymph nodes or liver metastases; and age > 80 years or the presence of other co-morbid conditions. Among these patients, metal stents were placed when they met the following conditions: the patient did not want an additional PDT due to the high cost, the patient was referred from a distant location; and there was a change to poor performance status during follow-up. We included patients with lymph node and liver metastasis but excluded those with extrahepatic distant metastasis whose prognoses are expected to be extremely poor.

Using these criteria, 199 patients were excluded because of surgery (n = 38), a plastic stent (n = 72), palliative chemotherapy or radiotherapy (n = 30), more than two sessions of PDT (n = 21), a Karnofsky performance status of less than 60 (n = 10), percutaneous biliary drainage only (n = 11), unavailability for follow-up (n = 4), refusal of endoscopic treatment (n = 12) and sudden death during diagnostic workup (n = 1). After these exclusions, 33 patients with unresectable hilar CC were included in this study. Eighteen patients were treated using uncovered metal stents after one session of PDT (PDT group) and 15 patients were treated by endoscopic metal stenting alone (control group). All patients underwent a standard pretreatment evaluation that included thin-section, contrast-enhanced, multiphase spiral computed tomography and/or magnetic resonance imaging of the abdomen. Tissue diagnosis was obtained by endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangioscopy (PTCS) or direct peroral cholangioscopy with biopsy and/or cytology. Patients with no histological confirmation were diagnosed as having malignant disease on the basis of clinical outcome during follow-up for at least 12 mo. All tumors were staged using the American Joint Committee (7th edition) on Cancer Staging criteria for perihilar bile duct cancer[7]. Clinical, laboratory, radiological, endoscopic, and histopathologic data were collected prospectively and analyzed retrospectively. The local institutional review board approved this study.

Photodynamic therapy

PDT was administered by methods described previously[8,9]. Briefly, patients received porfimer sodium (Photofrin Ⅱ, Axcan Pharma, Quebec, Canada) intravenously at a dose of 2 mg/kg 48-h before ERCP or PTCS. For light distribution, we used flexible cylindrical diffuser probes (BioLitec, Stirling, Scotland) mounted on a 400-μm quartz fiber with an active distal tip length of 2 cm. The light source was a diode laser system (Ceralas PDT 633; CeramOptec, Bonn, Germany) with a maximum power output of 2 W and a wavelength of 633 ± 3 nm. The power emitted by the diffuser tip was calibrated to 400 mW/cm before PDT was conducted using an integrating sphere power meter. The mean irradiation time was 452 s (range: 400-600 s), using a power density of 300-400 mW/cm and energy dose of 180-200 J/cm (of diffuser length). The PDT with PTCS was usually applicable for more advanced Bismuth type-Ⅲ lesions. To perform the percutaneous PDT procedure, a guide wire was first inserted through the stricture into the common bile duct. A 6-F guiding catheter was then inserted along the guide wire, after which the guide wire was removed. A diffuser fiber was then inserted into the guiding catheter and the stricture site was irradiated from the distal to the proximal region under cholangioscopy and fluoroscopy.

Peroral PDT with ERCP was performed in patients who had coagulopathy or refused percutaneous mo-

dality or bismuth Ⅱ and some of Ⅲ. To perform the peroral PDT, the preloaded catheter (catheter and PDT fiber) was advanced across the bile duct tumor using a 0.035-inch guide wire. The tip of the catheter was cut just below its metal marker, allowing the fiber to pass. Tumor segments were treated sequentially proximal to distal. In both percutaneous or peroral PDT, PDT was performed in both sides of the intrahepatic duct in Bismuth Ⅲ and Ⅳ strictures. In the case of multiple strictures, as many second branches as possible were treated. After PDT was performed, uncovered metal stents (MI tech, Seoul, South Korea) with diameters of 10 mm and stent lengths of 6 and 8 cm were inserted to ensure adequate decompression and bile drainage. In patients with percutaneous PDT, metal stent was inserted using the Rendezvous technique.

Definition of events and follow-up

The clinical outcome was evaluated according to the following parameters: (1) functional success, as measured by a decrease in the bilirubin level to less than 50% of the pretreatment value within 7 d; (2) early complications (occurring within 30 d); (3) late complications including stent malfunction; and (4) revision methods such as endoscopic retrograde biliary drainage or percutaneous transhepatic biliary drainage (PTBD). Stent patency was calculated as the interval between stent insertion and stent occlusion. Stent occlusion was defined as signs of jaundice or cholangitis (e.g., fever, tenderness in the right upper quadrant, and/or > 2-fold elevation of the total serum bilirubin above baseline after stent insertion).

Follow-up of symptoms and biochemical parameters was done at 1 mo intervals. Tumor markers for carbohydrate antigen (CA) 19-9 and abdominal computed tomography scans were done every 3 mo. ERCP was performed to confirm obstruction and to perform biliary decompression if a new episode of cholangitis occurred. Enrolled patients were followed until death. If the patient died with a patent stent, the time interval was recorded as censored data.

Statistical analysis

Statistical analysis were performed using SPSS software (ver. 18.0; SPSS Inc., Chicago, IL). Numerical data are presented as the median with range. Intergroup comparisons were performed using the Mann-Whitney *U* test. Estimates of probabilities of survival for the follow-up study and cumulative stent patency were calculated using the Kaplan-Meier method with the log-rank test. Data are presented as medians and 95% CI. For survival rates, all deaths related to CC and procedures were included, but deaths unrelated to CC were treated as censored patients. All of the data were analyzed using an intention-to-treat analysis. Intention-to-treat survival was calculated from the day of treatment until death or the last follow-up. *P* values ≤ 0.05 were deemed to indicate statistical significance.

Table 1 Patient characteristics *n* (%)

	PDT group (PDT with metal stenting) (*n* = 18)	Control group (metal stent only) (*n* = 15)
Age, yr (range)	65.6 (44-89)	67 (53-82)
Male sex	12 (66)	12 (80)
AJCC stage[1]		
Ⅱ	3 (17)	0 (0)
ⅢA/ⅢB	0 (0)/6(33)	3 (20)/5 (33)
ⅣA/ⅣB	7 (39)/2 (11)	1 (7)/6 (40)
Bismuth type		
Ⅱ	3 (17)	4 (27)
Ⅲ	5 (28)	7 (47)
Ⅳ	10 (55)	4 (27)
Lymph node metastasis	13 (72)	10 (67)
Liver metastasis	2 (11)	2 (17)
CA 19-9 (U/mL)	229 (0.9-4800)	480 (1.3-4800)
Pre-albumin (g/dL)	3.5 (2.1-4.5)	3.2 (2.0-4.3)
Bilirubin (mg/dL)		
Pre-treatment	8.5 (0.6-31.9)	12.3 (0.9-26.0)
Post-treatment	1.3 (0.5-14.3)	2.9 (0.3-26.0)
Successful drainage[2]	15 (83)	12 (86)
Histologic confirmation	14 (78)	10 (67)
Unilateral/bilateral stenting	10 (55)/8 (45)	11 (73)/4 (27)
Early complications of procedure	3 (17)	2 (13)
Stent malfunctions	14 (78)	12 (80)
Follow-up period (median, d)	437	230

[1]American Joint Committee Cancer (AJCC) staging 7th edition; [2]Decrease of bilirubin to less than 50% of the pretreatment value within 7 d. Data are expressed as medians (range). PDT: Photodynamic therapy; CA: Carbohydrate antigen.

RESULTS

Thirty-three patients (24 male), mean age 67.9 years (range: 44-89 years) were treated. A total of 18 (55%) patients received PDT (percutaneous PDT = 11, peroral PDT = 7) and metal stenting, whereas 15 (45%) underwent metal stenting alone. Baseline clinical and demographic profiles in the two treatment groups were similar, except for bismuth type Ⅳ (Table 1). There were more patients with bismuth type Ⅳ in the PDT group than in the control group (10/18 *vs* 4/15; *P* = 0.025). There were no significant differences between the groups with regard to age, gender, pre-procedure bilirubin, CA 19-9, stent length, or tumor stage. The diagnosis was confirmed histologically in 14 PDT patients (78%) and 10 control patients (67%). Histological confirmation of malignancy in PDT group was obtained by brush cytology in three ERCP patients and by intraductal biopsy examination in 11 PTCS patients. In the control group, histological confirmation was obtained by brush cytology in nine ERCP patients, and by intraductal biopsy in one direct cholangioscopy (using the mother-baby scope technique). Successful drainage was achieved in 83% and 86% of patients in PDT and control patients, respectively. No significant difference was observed in the degree of decrease of total bilirubin between the two groups (*P* = 0.2).

Stent patency and survival

The metal stent patency duration was longer in the PDT

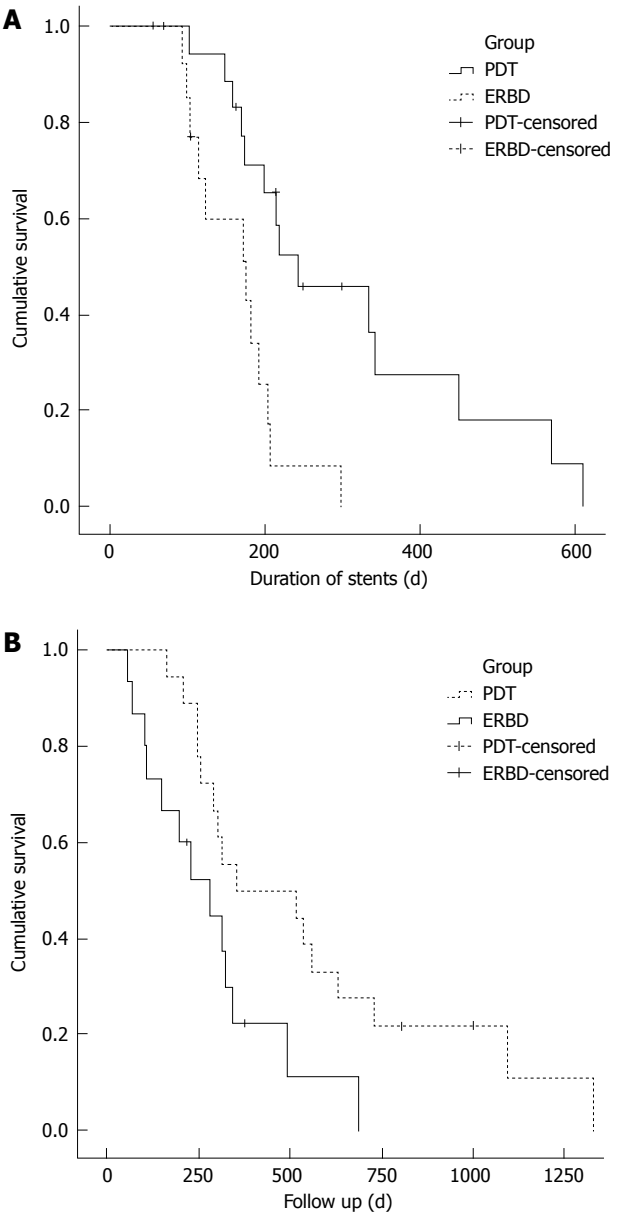


Figure 1 Kaplan-Meier analysis of metal stent patency rates and overall survival in the photodynamic therapy and control groups. A: Kaplan-Meier analysis of metal stent patency rates. The median stent patency was longer in the photodynamic therapy (PDT) group than in the control group (244 d *vs* 177 d; $P = 0.002$); B: Kaplan-Meier analysis of overall survival. The median survival in the PDT group was 356 d, *vs* 230 d in the stent-only group ($P = 0.006$). ERBD: Endoscopic retrograde biliary drainage.

group than in controls (Figure 1A). The median stent patency (range) was 244 ± 66 d (72-570 d) in the PDT group and 177 ± 45 d (70-309 d) in the controls ($P = 0.002$). Table 2 shows the results of univariate analysis of all variables associated with stent patency in all patients. PDT treatment ($P = 0.002$) was a significant predictive factor of longer stent patency on univariate analysis (Table 2). Factors including age, gender, bismuth type, pretreatment CA 19-9 and total bilirubin, T stage, number of endoprostheses (unilateral *vs* bilateral), and PDT method (PTCS *vs* ERCP) did not significantly affect stent patency according to univariate analysis.

Table 2 Univariate analysis of all prognostic factors associated with stent patency and patient survival in all patients

Variables	Cases	Univariate (Kaplan-Meier and log-rank test)			
		Median stent patency (d)	*P* value	Median patient survival (d)	*P* value
Age (yr)					
< 65 *vs* ⩾ 65	12:21	174:206	0.524	539:290	0.103
Gender					
Male *vs* female	24:9	193:298	0.278	322:303	0.766
CA 19-9 (U/mL)					
< 100 *vs* ⩾ 100	7:26	215:177	0.308	250:317	0.145
Pre-PDT bilirubin (mg/dL)					
< 3.0 *vs* ⩾ 3.0	15:18	244:181	0.112	493:250	0.026
T stage[1]					
T1,2 *vs* T3,4	11:22	174:207	0.388	317:283	0.527
Number of endoprotheses					
Unilateral *vs* bilateral	21:12	206:193	0.454	317:322	0.758
Bismuth type					
Ⅱ, Ⅲ *vs* Ⅳ	19:14	206:193	0.644	283:356	0.551
PDT method					
PTCS *vs* ERCP	11:22	200:334	0.075	322:214	0.621
Treatment group					
PDT *vs* ERBD	18:15	244:177	0.002	356:283	0.023

[1]American Joint Committee Cancer (AJCC) staging, 7th edition. PDT: Photodynamic therapy; PTCS: Percutaneous cholangioscopy; ERBD: Endoscopic retrograde biliary drainage; CA: Carbohydrate antigen; ERCP: Endoscopic retrograde cholangiopancreatography.

Compared with the controls, the PDT group was associated with longer patient survival (Figure 1B). The median survival time (range) was 356 ± 213 d (163-1330 d) in the PDT group and 230 ± 73 d (56-687 d) in the controls ($P = 0.006$). Table 2 shows the results of the univariate analysis of all variables considered. Statistically significant predictors of longer survival were PDT treatment and a lower total bilirubin level before the procedure.

Complications

Early complications were experienced by two control patients (13%) and three (17%) in the PDT group. In the control group, cholangitis occurred in 2 patients (13%), while in the PDT group, cholangitis and cholecystitis were each observed in one patient (6%). PDT-specific adverse events occurred in one patient in the PDT group, who experienced dermal phototoxicity. No stent migration occurred in either group.

Stent malfunctions occurred in 14 (78%) and 12 (80%) of the control and PDT patients, respectively. Among these 26 patients, 22 (85%) with tumor ingrowth were treated endoscopically with insertion of one or two plastic stents (10F) into unilateral or bilateral ducts through metal stents. In four patients with tumor recurrence, tumor ingrowth and overgrowth occurred in the hilar portion and more proximal intrahepatic duct, resulting in revisionary treatment with PTBD.

DISCUSSION

Hilar CC has an extremely poor prognosis, with an average five-year survival rate of 5%-10%. Surgery provides

the only possibility for a cure, but due to its anatomical location and natural history, the disease is locally advanced in most patients at the time of diagnosis. Therefore, effective palliation to alleviate symptoms associated with jaundice and the prevention of biliary sepsis are the fundamental goals for most patients with hilar CC[5]. Although relief of biliary obstruction by endoscopic placement of metal stents is regarded as an optimal palliative measure in hilar CC, the clinical course after even successful stent insertion is one of disease progression and death from liver failure or cholangitis within 4-9 mo[10]. This clinical course[1] is related to the ability to decompress affected proximal segments[2] and recurrent stent occlusion, because these stents are unable to remodel malignant tissues[11].

PDT is an evolving therapy for treatment of cancers that are resistant to standard oncologic treatment. PDT involves the injection of an intravenous photosensitizing drug followed by endoscopic application of light to the tumor bed. The interaction between light and the photoagent causes death of cancer cells and tumor thrombosis by generating oxygen free radicals. PDT is currently being used for cases of hilar cholangiocarcinoma[12,13]. Even in patients with advanced hilar CC, PDT has been shown to improve survival, quality of life, and to have a performance superior to that of biliary stenting in uncontrolled and randomized controlled trials[6,14-16]. In our study, Kaplan-Meier analysis demonstrated improved survival in the PDT group compared with the stent-only group (356 d *vs* 230 d, $P = 0.006$), in accordance with previous reports[11,14,17].

Effective palliation is essential, because biliary drainage and prevention of cholestasis are crucial for prevention of pruritus, cholangitis, and death in patients with hilar CC. The approach to palliative decompression has evolved from surgery and percutaneous to endoscopic management in order to prevent cholestasis and improve mortality. Endoscopy of hilar CCs is generally challenging and complex due to the involvement of multiple bile ducts requiring two or more stents; indeed, patency rates of endobiliary stents are lower than those of distal tumors[16,18,19]. Moreover, the efficacy of endoscopic stenting in a hilar CC is often limited by stent patency, which is related to proximal tumor obstruction, because the stent does not affect tissue remodeling, unlike benign conditions[11,20,21]. To address this issue, multiple studies have investigated the positive effects of the combination of bile duct stenting with PDT on patient survival[6,14,15]. However, a paucity of information exists regarding the effect of PDT on stent patency.

In our study, metal stent patency was longer in the PDT group than in the stent-only group. The median stent patency was 244 d in the PDT group and 177 d in the control group ($P = 0.002$). The main causes of obstruction of metal stents in bile ducts is tumor ingrowth or overgrowth[22]. PDT offers the possibility of tumor "remodeling", which can enhance or prolong the decompression effect[23]. Accepting this hypothesis, the ability of PDT to destroy cancer cells and lessen cholestasis may prolong stent patency. In this context, this study is meaningful because the longer stent patency that is achieved by PDT may diminish the need for further procedures, such as stent revision or percutaneous biliary drainage, improving the quality of life of hilar CC patients whose prognosis is poor.

This study was limited because of its retrospective nature and small sample size. Thus it may be not possible to reach statistical significance in terms of differences in overall survival between the groups. Because hilar CC is a rare malignancy and PDT is offered at few tertiary centers in South Korea, inclusion of sufficient patients to complete a well-designed palliative study is problematic. However, the study population was derived from a larger cohort of patients with CC, who were followed until death. Median survival of this cohort was comparable to that of other cohorts reported in the literature, which may decrease the possibility of significant selection bias.

In summary, metal stenting after one session of PDT may be safe with acceptable complication rates. The PDT group was associated with a significantly longer stent patency period and patient survival compared with the control group in patients with unresectable hilar CC. A prospective randomized multicenter study is required to confirm these data.

COMMENTS

Background

Although endoscopic biliary metal stenting is the mainstay of palliative treatment in patients with unresectable hilar cholangiocarcinoma (CC), tumor ingrowth or overgrowth in uncovered stents is a significant problem. The duration of metal stent patency for hilar CCs was shorter than that for the distal bile duct. Photodynamic therapy (PDT) is an evolving therapy for treatment of hilar CCs that are resistant to standard oncologic treatment.

Research frontiers

The authors observed that the one session of PDT with metal stenting was associated with a significantly longer stent patency period and patient survival compared with the metal stent only group in patients with unresectable hilar CC.

Innovations and breakthroughs

Metal stent patency was longer in the PDT group than in the stent-only group. The median stent patency was 244 d in the PDT group and 177 d in the control group ($P = 0.002$). The ability of PDT to destroy cancer cells and lessen cholestasis may prolong stent patency.

Applications

Just one session of PDT with endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangioscopy (PTCS) before metal stent placement is promising because it prolongs stent patency in unresectable hilar CC. A prospective randomized multicenter study is required to assess this technique.

Terminology

PDT is an emerging palliative strategy for unresectable hilar CC based on the intravenous administration of photosensitizing agents that preferentially accumulate in malignant cells. PDT is performed to the targeted area of bile ducts using ERCP or PTCS.

Peer review

PDT in patients with CC has been reported as effective palliative treatment providing improvement in cholestasis and quality of life, and prolonging survival. The authors conclude that metallic stenting after PDT may be a safe, acceptable modality associated with longer stent patency in patients with unresectable hilar CC.

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BRIEF ARTICLE

Surgical outcome of pancreatic cancer using radical antegrade modular pancreatosplenectomy procedure

Ye Rim Chang, Sung-Sik Han, Sang-Jae Park, Seung Duk Lee, Tae Suk Yoo, Young-Kyu Kim, Tae Hyun Kim, Sang Myung Woo, Woo Jin Lee, Eun Kyung Hong

Ye Rim Chang, Department of Surgery, Seoul National University Hospital, Seoul 110-744, South Korea
Sung-Sik Han, Sang-Jae Park, Seung Duk Lee, Tae Suk Yoo, Young-Kyu Kim, Tae Hyun Kim, Sang Myung Woo, Woo Jin Lee, Eun Kyung Hong, Center for Liver Cancer, National Cancer Center, Gyeonggido 411-764, South Korea
Author contributions: Han SS designed the research; Park SJ, Lee SD, Yoo TS and Kim YK performed the research; Kim TH, Woo SM and Lee WJ provided discussion of the chemotherapy and radiotherapy; Hong EK reviewed the slide; Chang YR analyzed data and wrote the paper.
Correspondence to: Sung-Sik Han, MD, PhD, Center for Liver Cancer, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang-si, Gyeonggido 411-764, South Korea. sshan@ncc.re.kr
Telephone: +82-31-9201641 Fax: +82-31-9201138
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Abstract

AIM: To evaluate the surgical outcomes following radical antegrade modular pancreatosplenectomy (RAMPS) for pancreatic cancer.

METHODS: Twenty-four patients underwent RAMPS with curative intent between January 2005 and June 2009 at the National Cancer Center, South Korea. Clinicopathologic data, including age, sex, operative findings, pathologic results, adjuvant therapy, postoperative clinical course and follow-up data were retrospectively collected and analyzed for this study.

RESULTS: Twenty-one patients (87.5%) underwent distal pancreatectomy and 3 patients (12.5%) underwent total pancreatectomy using RAMPS. Nine patients (37.5%) underwent combined vessel resection, including 8 superior mesenteric-portal vein resections and 1 celiac axis resection. Two patients (8.3%) underwent combined resection of other organs, including the colon, stomach or duodenum. Negative tangential margins were achieved in 22 patients (91.7%). The mean tumor diameter for all patients was 4.09 ± 2.15 cm. The 2 patients with positive margins had a mean diameter of 7.25 cm. The mean number of retrieved lymph nodes was 20.92 ± 11.24 and the node positivity rate was 70.8%. The median survival of the 24 patients was 18.23 ± 6.02 mo. Patients with negative margins had a median survival of 21.80 ± 5.30 mo and those with positive margins had a median survival of 6.47 mo (P = 0.021). Nine patients (37.5%) had postoperative complications, but there were no postoperative mortalities. Pancreatic fistula occurred in 4 patients (16.7%): 2 patients had a grade A fistula and 2 had a grade B fistula. On univariate analysis, histologic grade, positive tangential margin, pancreatic fistula and adjuvant therapy were significant prognostic factors for survival.

CONCLUSION: RAMPS is a feasible procedure for achieving negative tangential margins in patients with carcinoma of the body and tail of the pancreas.

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Key words: Carcinoma; Pancreas; Surgical resection; Survival; Radical antegrade modular pancreatosplenectomy

Peer reviewer: Hiroki Yamaue, MD, Professor and Chairman, Second Department ofSurgery, Wakayama Medical University School of Medicine, 811-1 Kimiidera, Wakayama 641-8510, Japan

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INTRODUCTION

Distal pancreatectomy, originally described at the Mayo Clinic in 1913, has been the standard procedure for carcinoma of the body or tail of the pancreas[1]. This traditional approach is, however, associated with a high tangential margin positive rate and is not based on the physiologic lymphatic drainage of the pancreas[2]. So far, there has been little progress in overcoming these shortcomings. Radical antegrade modular pancreatosplenectomy (RAMPS), which was first introduced by Strasberg *et al*[3] in 2003 as a surgical treatment for carcinomas of the body and tail of the pancreas, is known as a feasible procedure for achieving a higher rate of negative tangential margins than the traditional approach. RAMPS is performed in a right-to-left fashion after early ligation of blood vessels, whereas pancreatosplenectomy is performed in a left-to-right fashion. In fact, RAMPS improves the tangential margin negative rate and provides better surgical exposure, especially in obese patients[3].

Strasberg *et al*[2] reported that negative tangential margins were obtained in 91% of all patients and that the 5-year overall survival rate was 26%. However, there have been few studies on the clinical outcomes following RAMPS[4]. The purpose of this study is to evaluate the surgical outcomes following RAMPS for carcinoma of the body and tail of the pancreas.

MATERIALS AND METHODS

Patients

Twenty-four patients underwent RAMPS with curative intent between January 2005 and June 2009 at the National Cancer Center in South Korea. History-taking, physical examination, liver function tests, tumor marker (carbohydrate antigen 19-9) levels, and abdominal computed tomography (CT) scans were used for diagnostic and staging workup. A whole-body positron emission tomography scan was added when necessary. Clinicopathologic data, including age, sex, operative findings, pathologic results, adjuvant therapy, postoperative clinical course and follow-up data were collected and analyzed retrospectively. A pancreatic fistula after surgery was defined based on the International Study Group of Pancreatic Fistula definition[5]: a drain output of any measurable volume on or after postoperative day 3 with an amylase concentration greater than 3 times the serum amylase concentration. Postoperative complications were reviewed and graded using the Clavien-Dindo classification[6].

Operative procedures

The operation was performed according to the procedure introduced by Strasberg *et al*[2]. After dissection of the gastro-colic ligament, we entered the lesser omentum and dissected the celiac axis, hepatic artery, and trunk of the splenic artery to divide the splenic artery. The neck of the pancreas was divided using electrocautery, and the pancreatic duct stump was ligated. After we divided the neck of the pancreas, the vertical plane of dissection reached the level of the aorta where the left renal vein was exposed. The left adrenal vein was ligated and divided when a posterior RAMPS was performed. During lymph node dissection, we removed regional nodes along the common hepatic and celiac artery. The soft tissue to the left of the hepatoduodenal ligament and superior mesenteric artery was also dissected. Lymph nodes along the splenic artery and splenic hilum were removed en-bloc with the specimen. Removal of the nerve plexus and lymph nodes was performed in the same manner (Figure 1).

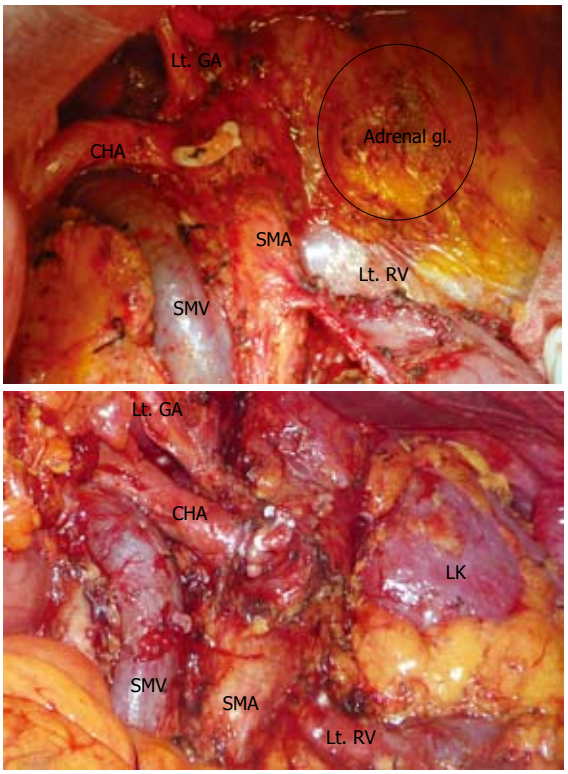


Figure 1 Photographs of operative field. A: Anterior radical antegrade modular pancreatosplenectomy (RAMPS); B: Posterior RAMPS. Lt. GA: Left gastric artery; CHA: Common hepatic artery; SMA: Superior mesenteric artery; SMV: Superior mesenteric vein; Lt. RV: Left renal vein; LK: Left kidney; gl: Gland.

Adjuvant therapy

We performed adjuvant concurrent chemoradiation therapy (CCRT) on all patients without severe medical comorbidities or poor physical status. Several studies have demonstrated the positive effects of CCRT[7-17]. Twenty patients (83.3%) underwent adjuvant CCRT using multiple-field techniques. The initial irradiated field, which was defined as the tumor bed plus regional nodes, received 45 Gy in 25 fractions using a 4-field technique (anteroposterior, posteroanterior and paired laterals) with 15-MV X-rays. The tumor bed boost field received an additional 5.4-10.8 Gy in 3 to 6 fractions of 1.8 Gy. Concomitant 5-fluorouracil-based chemotherapy during radiotherapy was given to all patients. Patients were followed every 3 mo with CT scans and tumor marker levels to detect recurrent disease.

Table 1 Pathological results

Parameters	*n* (%)
Mean tumor diameter	4.09 ± 2.15 cm
Tumor diameter in negative margin (*n* = 22)	3.80 ± 1.73 cm
Tumor diameter in positive margin (*n* = 2)	7.25 cm
Histologic grade	
Well-differentiated	2 (8.3)
Moderately-differentiated	17 (70.8)
Poorly-differentiated	5 (20.8)
T stage	
T2	3 (12.5)
T3	21 (87.5)
Lymph node metastasis	
(+)	17 (70.8)
(-)	7 (29.2)
Vascular invasion	
(+)	12 (50)
(-)	12 (50)
Lymphatic invasion	
(+)	15 (62.5)
(-)	9 (37.5)
Perineural invasion	
(+)	21 (87.5)
(-)	3 (12.5)
Tangential margin	
(+)	2 (8.3)
(-)	22 (91.7)

Table 2 Postoperative complications

Complications	*n* (%)	Grade (number)
Pancreatic fistula	4 (16.7)	A (2), B (2)[1]
Wound dehiscence	2 (8.3)	IIIa (2)[2]
Intraabdominal fluid collection	1 (4.2)	IIIa (1)[2]
Pleural effusion	1 (4.2)	IIIa (1)[2]
PV-SMV thrombosis	1 (4.2)	IIIa (1)[2]

[1]Graded by the International Study Group on Pancreatic Fistula classification; [2]Graded by Clavien-Dindo classification. PV: Portal vein; SMV: Superior mesenteric vein.

Statistical analysis

The χ^2 test, the independent *t* test, the Kaplan-Meier method with the log-rank test and a Cox regression model were used for statistical analysis. A *P* value of less than 0.05 was considered significant. SPSS® version 19.0 (Chicago, IL, United States) was used for all statistical analyses. This study was approved by the Institutional Review Board of the National Cancer Center in South Korea.

RESULTS

Operative findings and histopathologic results

The mean age of the total 24 patients was 60.00 ± 7.79 years with a male:female ratio of 1.18:1. The mean operative time was 305.42 ± 155.68 min. One patient required a blood transfusion during surgery. Three patients (12.5%) who had cancer in the body of the pancreas with diffuse infiltration into the head and tail were converted to a total pancreatectomy. Nine patients (37.5%) underwent combined vessel resection, including 8 superior mesenteric-portal vein (SMV-PV) resections and 1 celiac axis resection. Two patients (8.3%) underwent combined resection of other organs, including the colon, stomach or jejunum. Posterior RAMPS, including resection of the adrenal gland, was performed in 5 patients (20.8%).

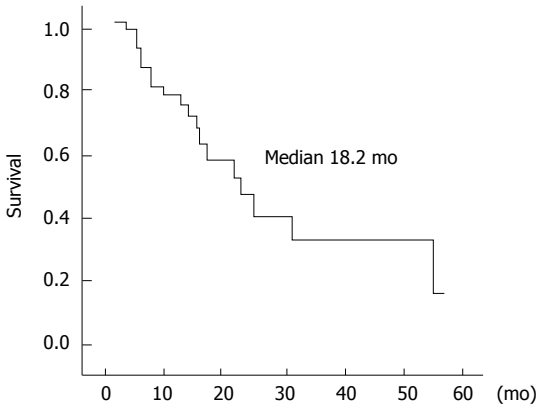


Figure 2 Overall survival curve of the 26 patients.

Histopathologic examination showed that 21 cases (87.5%) were T3. As for histologic grade, moderately differentiated adenocarcinoma was found in 17 patients (70.8%). The mean number of retrieved lymph nodes was 20.92 ± 11.24 and node positivity was observed in 17 patients (70.8%). The pancreatic parenchymal resection margin was negative for all patients with a mean distance of 16.52 ± 13.8 mm. Negative tangential margins were obtained in 22 patients (91.7%). The mean tumor diameter of all patients was 4.09 ± 2.15 cm. Two patients with positive tangential margins had a mean tumor diameter of 7.3 cm (Table 1). Among 9 patients with combined vessel resection, 1 patient had no evidence of vascular invasion on pathology.

Nine patients (37.5%) had postoperative complications (Table 2). Pancreatic fistula occurred in 4 patients (16.7%): 2 patients had a grade A fistula and 2 had a grade B fistula. Intra-abdominal fluid collections and grade B pancreatic fistulae were treated with percutaneous drainage and intravenous antibiotics. Patients with PV-SMV thrombosis were treated with percutaneous thrombectomy and stenting. There were no postoperative mortalities.

Survival and prognostic factors

In this study, the median survival was 18.23 ± 6.02 mo with a median follow-up period of 20.06 ± 14.46 mo (Figure 2). Twenty-one patients (87.5%) had recurrence at follow-up. Two patients (8.3%) had local recurrence, 14 patients (58.3%) had distant metastasis, and 5 patients (20.8%) had both.

Celiac axis resection (Appleby operation) was performed in 1 patient who died of recurrent disease 12.5 mo after surgery. Three patients who underwent total pancreatectomy had a median survival of 21.23 ± 4.25 mo which was similar to the median survival of all patients in this study. One patient was free of cancer at his last

Table 3 Prognostic factors by univariate analysis

Factors		*n*	Median survival (mo)	*P* value
Age	≤ 60 yr	11	21.84 ± 12.90	0.249
	> 60 yr	13	16.93 ± 8.54	
Gender	Male	13	21.80 ± 6.84	0.995
	Female	11	18.23 ± 4.77	
Transfusion	(+)	1	26.1	0.345
	(-)	23	19.79 ± 14.73	
Operative time	≤ 300 min	18	21.80 ± 7.42	0.534
	> 300 min	6	12.53 ± 6.96	
Type of RAMPS	Anterior	19	21.80 ± 4.34	0.421
	Posterior	5	12.53 ± 6.65	
Combined vascular resection	(+)	9	16.93 ± 5.48	0.333
	(-)	15	26.27 ± 10.81	
Tumor size	≤ 4 cm	15	26.27 ± 9.44	0.862
	> 4 cm	9	18.23 ± 4.91	
Histologic grade	WD	2	16.93	0.001
	MD	17	26.27 ± 5.87	
	PD	5	6.46 ± 1.06	
T-stage	2	3	21.17 ± 10.94	0.448
	3	21	18.23 ± 5.79	
N-stage	0	7	21.80 ± 16.32	0.485
	1	17	18.23 ± 4.70	
Tangential margin	(+)	2	6.46	0.031
	(-)	22	21.80 ± 5.30	
Complication	(+)	9	12.53 ± 4.77	0.385
	(-)	15	21.80 ± 4.05	
Pancreatic fistula	(+)	4	6.46 ± 1.52	0.003
	(-)	20	26.27 ± 6.49	
Adjuvant therapy	(+)	20	26.27 ± 6.49	< 0.001
	(-)	4	6.30 ± 0.48	

RAMPS: radical antegrade modular pancreatosplenectomy; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated.

visit, while the other 2 patients had local or systemic recurrence.

Twenty patients (83.3%) underwent adjuvant CCRT. CCRT was also performed in patients with pancreatic fistula with the exception of 1 patient with grade A pancreatic fistula who was not a candidate because of age.

On univariate analysis, histologic grade, positive tangential margin, pancreatic fistula and adjuvant therapy were significant prognostic factors for survival (Table 3). Unfortunately, this study was not powered to show significant factors on multivariate analysis.

DISCUSSION

Because carcinomas of the body and tail of the pancreas are often found in a larger size than those of the head, unresectable cases are more common and the recurrence rate after resection is also higher[18-22]. The goals of pancreatic cancer surgery are to obtain tumor-free margins and perform a sufficient regional lymphadenectomy. However, conventional distal pancreatectomy, which is performed in the left-to-right direction along the anterior border of Gerota's facia, is inappropriate for achieving this goal because the tumor easily infiltrates the retroperitoneum and spreads to lymph nodes at an early stage. There have been few surgical methods to overcome these limitations. Yang *et al*[23] introduced retrograde distal pancreatectomy, which cuts the neck of the pancreas first and proceeds with dissection in the right-to-left direction. This procedure is a useful method for exposing the portal-superior mesenteric vein junction, which helps to avoid operative injuries. RAMPS was introduced with the theoretical advantages of obtaining a higher rate of negative tangential margins and a higher lymph node count. Therefore, we applied RAMPS to all distal pancreatectomy cases.

The margin-negative rate of RAMPS reported by Strasberg *et al*[2] and our institute was 91% and 91.7%, respectively, which is higher than the margin-negative rate of conventional distal pancreatectomy (70% to 80%)[5,7,8]. In our study, only 2 patients showed positive resection margins. These 2 patients had a lower median survival than patients with negative margins (6.47 mo *vs* 21.80 ± 5.30 mo, $P = 0.03$). The first patient with a positive tangential margin underwent posterior RAMPS and was stage T3N1 with a tumor size of 10.5 cm. He received adjuvant CCRT after recovering from grade B pancreatic fistula, but had multiple liver metastases at follow-up and survived 6.5 mo. The second patient underwent anterior RAMPS with combined jejunal resection due to gross invasion. He had stage T3N0 disease with a tumor size of 4.0 cm. He had both local recurrence and distant metastasis after adjuvant CCRT and survived 19.0 mo.

RAMPS has also been applied to laparoscopic distal pancreatectomy because it provides good surgical exposure for performing a right-to-left distal pancreatectomy. Kang *et al*[24] and Choi *et al*[25] reported outcomes of laparoscopic distal pancreatectomy performed on well-selected patients with left-sided pancreatic ductal adenocarcinomas.

In a previous study performed by Strasberg *et al*[2], the mean number of retrieved lymph nodes and lymph node positivity rate were 14.3 and 48%, respectively. In our study, the mean number of retrieved lymph nodes was 20.92 ± 11.24 and the lymph node positivity rate was 70.8%. Lymph node status is known to be a very important prognostic factor in pancreatic cancer. Some previous reports emphasized the lymph node ratio (the number of positive lymph nodes/the total number of retrieved lymph nodes) in pancreatic cancer[26]. However, few studies have mentioned the number of retrieved lymph nodes, which makes it difficult to compare the surgical efficacy of RAMPS with conventional distal pancreatectomy.

Strasberg *et al*[2] reported that the median survival time and the 5-year survival rate after RAMPS were 21 mo and 26%, respectively, which were better than those of previous reports[18]. It is known that patients with left-sided pancreatic cancer have poorer survival rates than patients with right-sided pancreatic cancer. However, surgical outcomes for patients with left-sided cancer were better than those with right-sided pancreatic cancer. Our patients had a median survival of 18.23 ± 6.02 mo, which was a little better than the survival following distal pancreatectomy reported by previous studies of conven-

tional distal pancreatectomy. However, our patients were mostly T3 or higher (87.5%), and lymph node positivity was 70.8%, which means that most of our patients were advanced stage. When viewed in this light, our results are better than those reported by previous studies and are comparable with those of right-sided pancreatic cancer.

We had 19 patients (79.2%) with systemic recurrence. Therefore, even though RAMPS has surgical advantages in terms of local control, there are limitations in preventing cancer progression, especially through systemic recurrence.

We performed distal pancreatectomy for carcinomas of the body and tail of the pancreas using RAMPS, with a negative tangential margin of 91.7%, no mortality and acceptable morbidity. It is suggested that RAMPS is a safe and feasible procedure for carcinomas of the body and tail of the pancreas. Further studies with larger sample sizes are needed to confirm our results.

COMMENTS

Background

Radical antegrade modular pancreatosplenectomy (RAMPS) is known as a feasible procedure for achieving a higher rate of negative tangential margins than the traditional approach. Although RAMPS is widely performed, there have been few studies on its clinical outcomes.

Applications

This study provides reference data for future larger studies.

Terminology

RAMPS was first introduced in 2003 as a surgical treatment for carcinomas of the body and tail of the pancreas.

Peer review

RAMPS seems to be a feasible procedure for achieving negative tangential margins for patients with carcinoma of the body and tail of the pancreas.

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BRIEF ARTICLE

Biochemical characteristics of neonatal cholestasis induced by citrin deficiency

Jian-She Wang, Xiao-Hong Wang, Ying-Jie Zheng, Hai-Yan Fu, Rui Chen, Yi Lu, Ling-Juan Fang, Takeyori Saheki, Keiko Kobayashi

Jian-She Wang, Department of Pediatrics, Jinshan Hospital, Fudan University, Shanghai 200540, China
Jian-She Wang, Xiao-Hong Wang, Hai-Yan Fu, Rui Chen, Yi Lu, Ling-Juan Fang, The Center for Pediatric Liver Diseases, Children's Hospital of Fudan University, Shanghai 201102, China
Ying-Jie Zheng, Department of Epidemiology, School of Public Health, Fudan University, Shanghai 200032, China
Takeyori Saheki, Institute of Resource Development and Analysis, Kumamoto University, Kumamoto 860-0811, Japan
Keiko Kobayashi, Department of Molecular Metabolism and Biochemical Genetics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan
Author contributions: Wang JS, Wang XH, Zheng YJ and Fu HY contributed equally to this paper; Wang JS proposed the idea and wrote the first draft of this paper; Zheng YJ contributed to the statistical analysis and created all the figures; Fu HY contributed to the data collection; Wang JS, Fu HY, Chen R, Saheki T and Kobayashi K contributed to the gene tests and Western blotting; Wang JS, Wang XH and Lu Y contributed to patient management; and all the authors contributed to the design, data analysis, draft revision and final approval of the paper for publishing.
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Correspondence to: Jian-She Wang, Professor, Department of Pediatrics, Jinshan Hospital, Fudan University, Shanghai 200540, China. jshwang@shmu.edu.cn
Telephone: +86-21-64931171 Fax: +86-21-64931171
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Abstract

AIM: To explore differences in biochemical indices between neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and that with other etiologies.

METHODS: Patients under 6 mo of age who were referred for investigation of conjugated hyperbilirubinaemia from June 2003 to December 2010 were eligible for this study. After excluding diseases affecting the extrahepatic biliary system, all patients were screened for the two most common *SLC25A13* mutations; the coding exons of the entire *SLC25A13* gene was sequenced and Western blotting of citrin protein performed in selected cases. Patients in whom homozygous or compound heterozygous *SLC25A13* mutation and/or absence of normal citrin protein was detected were defined as having NICCD. Cases in which no specific etiological factor could be ascertained after a comprehensive conjugated hyperbilirubinaemia work-up were defined as idiopathic neonatal cholestasis (INC). Thirty-two NICCD patients, 250 INC patients, and 39 infants with cholangiography-confirmed biliary atresia (BA) were enrolled. Laboratory values at their first visit were abstracted from medical files and compared.

RESULTS: Compared with BA and INC patients, the NICCD patients had significantly higher levels of total bile acid (TBA) [all measures are expressed as median (inter-quartile range): 178.0 (111.2-236.4) μmol/L in NICCD *vs* 112.0 (84.9-153.9) μmol/L in BA and 103.0 (70.9-135.3) μmol/L in INC, $P = 0.0001$]. The NICCD patients had significantly lower direct bilirubin [D-Bil 59.6 (43.1-90.9) μmol/L in NICCD *vs* 134.0 (115.9-151.2) μmol/L in BA and 87.3 (63.0-123.6) μmol/L in INC, $P = 0.0001$]; alanine aminotransferase [ALT 34.0 (23.0-55.0) U/L in NICCD *vs* 108.0 (62.0-199.0) U/L in BA and 84.5 (46.0-166.0) U/L in INC, $P = 0.0001$]; aspartate aminotransferase [AST 74.0 (53.5-150.0) U/L in NICCD *vs* 153.0 (115.0-239.0) U/L in BA and 130.5 (81.0-223.0) U/L in INC, $P = 0.0006$]; albumin [34.9 (30.7-38.2) g/L in NICCD *vs* 38.4 (36.3-42.2) g/L in BA and 39.9 (37.0-42.3) g/L in INC, $P = 0.0001$]; glucose [3.2 (2.0-4.4) mmol/L in NICCD *vs* 4.1 (3.4-5.1) mmol/L in BA and 4.0 (3.4-4.6) mmol/L in INC, $P = 0.0014$] and total cholesterol [TCH 3.33 (2.97-4.00) mmol/L in NICCD *vs* 4.57 (3.81-5.26) mmol/L in BA and 4.00 (3.24-4.74) mmol/L in INC, $P = 0.0155$] levels. The D-Bil to total bilirubin (T-Bil) ratio was significantly lower in NICCD patients [all measures

are expressed as median (inter-quartile range): 0.54 (0.40-0.74)] than that in BA patients [0.77 (0.72-0.81), P = 0.001] and that in INC patients [0.74 (0.59-0.80), P = 0.0045]. A much higher AST/ALT ratio was found in NICCD patients [2.46 (1.95-3.63)] compared to BA patients [1.38 (0.94-1.97), P = 0.0001] and INC patients [1.48 (1.10-2.26), P = 0.0001]. NICCD patients had significantly higher TBA/D-Bil ratio [3.36 (1.98-4.43) *vs* 0.85 (0.72-1.09) in BA patients and 1.04 (0.92-1.14) in INC patients, P = 0.0001], and TBA/TCH ratio [60.7 (32.4-70.9) *vs* 24.7 (19.8-30.2) in BA patients and 24.2 (21.4-26.9) in INC patients, P = 0.0001] compared to the BA and INC groups.

CONCLUSION: NICCD has significantly different biochemical indices from BA or INC. TBA excretion in NICCD appeared to be more severely disturbed than that of bilirubin and cholesterol.

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Key words: Cholestasis; Biliary atresia; Infants; Idiopathic neonatal cholestasis; *SLC25A13*

Peer reviewer: Dr. Stefan Wirth, Professor, Children's Hospital, Heusnerstt. 40, 42349 Wuppertal, Germany

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INTRODUCTION

Citrin deficiency, caused by mutations in the *SLC25A13* gene on chromosome 7q21.3, is an autosomal recessive disease that was first discovered in Japan and thereafter identified worldwide[1-8]. At least two main phenotypes of citrin deficiency have been established: neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, OMIM #605814)[9-11] and adult-onset type Ⅱ citrullinemia (CTLN2, OMIM #603471)[1,12]. The clinical features and diagnostic criteria of CTLN2 have been well established, but those of NICCD have not yet been established.

Children with NICCD usually have transient intrahepatic cholestasis that disappears by the age of 1 year with appropriate management[13]. However, some patients need liver transplantation or may die from the disease during infancy[14-17]. Others may develop severe CTLN2 symptoms unexpectedly one to several decades later[13]. Prompt detection and specific lactose-free and/or medium-chain triglyceride formula may contribute to the avoidance of a complicated course in the NICCD phase. However, the prompt diagnosis of NICCD is still a challenge because the clinical features of cholestasis induced by citrin deficiency are presently not fully understood[18].

Some biochemical indices, including total bilirubin (T-Bil), direct bilirubin (D-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (GGT), and α-fetoprotein (AFP) have been analyzed or compared between patients with NICCD, biliary atresia (BA) or idiopathic neonatal cholestasis (INC), with significant differences found between them[4]. However, comparisons of serum total bile acid (TBA) and total cholesterol (TCH) levels between different causes of neonatal cholestasis has rarely been reported before[19].

Bilirubin, bile acids and cholesterol are all mainly physiologically excreted by the hepatobiliary system. Usually, the blood levels of all these compounds increase in the event of blockage of the extrahepatic biliary system. However, they may be affected to different extents in intrahepatic cholestasis with different etiology. For instance, blood TCH and TBA levels are usually elevated significantly in Alagille syndrome, even in cases in which the bilirubin level is only mildly elevated; however, the blood TCH level is usually normal in cases of progressive familial intrahepatic cholestasis type 1 or 2 despite significant elevation of blood TBA and bilirubin levels. Therefore, comparison of these biochemical indices in neonatal cholestasis cases with different etiologies will enable better characterization of the biochemical changes of the disease and may help elucidate the mechanism of cholestasis caused by citrin deficiency.

Therefore, the aims of this study were to explore the differences in biochemical indices, including D-Bil, TBA, TCH and their ratios between cholestasis with different etiologies, and to explore the mechanism of cholestasis caused by citrin deficiency.

MATERIALS AND METHODS

Subjects

Patients under 6 mo of age who were referred to the Children's Hospital of Fudan University, a tertiary referral hospital and primary specialized paediatric hospital in Eastern China, from June 2003 to December 2010, for investigation of conjugated hyperbilirubinaemia were eligible for this study. Conjugated hyperbilirubinemia was defined as serum T-Bil levels > 75 μmol/L, with a conjugated fraction accounting for > 20% of the total, or having conjugated bilirubin levels > 17 μmol/L with serum T-Bil levels < 75 μmol/L[20]. Patients who had obvious extrahepatic abnormalities or prolonged prothrombin time that could not be corrected by parenteral administration of vitamin K1 were excluded. Subjects who satisfied the above criteria as well as the following specific criteria for each of the three groups were included.

BA group: Patients with neonatal cholestasis in whom no isotope excretion was demonstrated by hepatobiliary iminodiacetic acid (HIDA) scintigraphy, and in whom diagnosis of BA was confirmed by laparoscopic or laparotomic cholangiography were eligible for this group.

INC group: Intrahepatic cholestasis was defined as con-

Table 1 Differential diagnosis have been excluded

Affecting bile duct	Infectious	Metabolic	Others
Biliary atresia	Herpes viruses	α-1 antitrypsin deficiency	Endocrinological
Choledochal cyst	Rubella virus	Neonatal iron storage disease	Hypothyroidism
Cholelithiasis	Enteroviruses	Amino acid disorders	Panhypopituitarism/septo-optic dysplasia
Inspissated bile	Hepatitis viruses	Tyrosinemia	Genetic
Tumor	Human immunodeficiency virus	Hypermethioninemia	ATP8B1 deficiency
Hemangioma	Syphilis	Mevalonate kinase dificiency	ABCB11 deficiency
Bile duct stenosis/stricture/perforation	Toxoplasmosis	Glucogen storage diseases	ABCB4 deficiency
Neonatal sclerosing cholangitis	Bacterial sepsis	Gaucher disease	Bile acid synthetic defects
Caroli disease	Urinary tract infections	Niemann–Pick disease	Neonatal Dubin–Johnson syndrome
Alagille syndrome		Wolman disease	Various trisomies
		Zellweger syndrome	Arghrogryposis
		Infantile Refsum disease	Hematological
		Mitochondrial disorders	Hemophagocytic lymphohistiocytosis
		Urea cycle disorders	Langerhans cell histiocytosis
			Miscellaneous drug effects
			Total parenteral nutrition

jugated hyperbilirubinemia following the exclusion of diseases affecting the extrahepatic biliary system (Table 1) by imaging of the hepatobiliary system. The imaging procedures included an ultrasound scan and HIDA scintigraphy in each case and laparotomic/laparoscopic cholangiography in selected cases. Idiopathic neonatal cholestasis (INC) was defined when no specific etiological factor (Table 1) could be ascertained after a comprehensive conjugated hyperbilirubinemia test[21]. Patients with at least single-allele mutation of *SLC25A13* gene were excluded from this group as well.

NICCD group: The strategy of testing for *SLC25A13* gene mutations in intrahepatic cholestatic infants had been reported previously[22,23]. All intrahepatic cholestasis infants with unknown causes were screened for the two most common mutations of the *SLC25A13* gene in Chinese, 851del4 and 1638ins23. For patients with various aminoacidemia or patients with only single-allele mutation who were found by the above screening method, the entire 18 coding exons together with the flanking sequence of the *SLC25A13* gene were amplified by polymerase chain reaction and directly sequenced. Western blotting analysis of citrin protein was performed on patients with biopsied liver specimens available. Only patients in whom homozygous or compound heterozygous *SLC25A13* gene mutation and/or absence of normal citrin protein were demonstrated, for whom a definite diagnosis of citrin deficiency could be made, were regarded as NICCD patients in this study. Patients with a probable diagnosis of citrin deficiency, that is, in whom there was only a heterozygous *SLC25A13* gene mutation and in whom absence of normal citrin protein could not be demonstrated by Western blotting were excluded.

Retrospective analyses of biochemical indices

The medical files of the patients who satisfied the above inclusion and exclusion criteria were reviewed following the approval of the Institute's Ethics Review Committee. Sex, birth weight, gestation age or term/preterm, age at which conjugated jaundice was first noticed, and the biochemical indices at presentation, were abstracted. Liver function tests and other routine laboratory data were obtained using standard methods.

Statistical analysis

Statistical analysis was performed using Stata/SE 10.0 for Windows (StataCorp LP, College Station, TX, United States of America). The descriptive data of the quantitative variables were reported in box-whisker plots and compared using Kruskal-Wallis rank tests among the three groups. For results with overall statistical significance, a Mann-Whitney test with a Bonferroni correction was further performed to test the medians between a series of pairwise groups. All *P* values were two-sided. Results were considered statistically significant at the 0.05 level.

RESULTS

Basic information

In total, 32 patients (19 male and 13 female) with a definite diagnosis of citrin deficiency were included in the NICCD group. Thirty-nine patients (24 male and 15 female) were included in the BA group. Two hundred and fifty patients (174 male and 76 female) were included in the INC group. The birth weight and the days at which conjugated jaundice was first noticed in the three groups are illustrated in Figure 1 or Table 2. The median birth weight was lowest in the NICCD group, but the difference did not reach statistical significance (Table 2). Conjugated jaundice was noticed earlier in the BA group compared with the INC group ($P < 0.05$, Figure 1A).

Comparison of biochemical indices among three groups

The biochemical data of the three groups were compared (Figure 1B-E, Table 2). The NICCD group had significantly lower ALT, AST, total protein, albumin, and glucose levels compared with the BA and INC groups, suggesting that the synthetic function and glucose metabolism were more severely damaged in the NICCD

Table 2 Comparison of birth weight, biochemical indices and their ratios among the three groups

	Reference range and unit	BA (n = 39)		INC (n = 39)		NICCD (n = 32)	
		Median	Inter-quartile	Median	Inter-quartile	Median	Inter-quartile
Birth weight	2.5-4.0 kg	3.2	2.9-3.8	3.1	2.8-3.5	2.9	2.4-3.4
Biochemical indices							
T-Bil[ac]	2-20 mmol/L	159.5	140.2-201.4	133.8	90.0-190.4	112.7	64.4-165.4
D-Bil[ace]	0-6 mmol/L	134	115.9-151.2	87.3	63.0-123.6	59.6	43.1-90.9
ALT[ce]	< 40 IU/L	108	62.0-199.0	84.5	46.0-166.0	34	23.0-55.0
AST[ce]	< 40 IU/L	153	115.0-239.0	130.5	81.0-223.0	74	53.5-150.0
GGT[ac]	< 50 IU/L	558	300.0-1086.0	155	91.0-294.0	187.5	136.0-253.0
TBA[ce]	< 40 mmol/L	112	84.9-153.9	103	70.9-135.3	177.9	111.2-236.4
Total protein[ce]	55-78 g/L	57.4	55.3-63.1	57.2	52.5-62.8	48.5	44.5-53.9
Albumin[ce]	35-55 g/L	38.4	36.3-42.2	39.9	37.0-42.3	34.9	30.7-38.2
Glucose[ce]	3.9-5.9 mmol/L	4.1	3.4-5.1	4	3.4-4.6	3.2	2.0-4.4
TCH[ce]	3.12-5.20 mmol/L	4.57	3.81-5.26	4	3.24-4.74	3.33	2.97-4.00
Ratios							
D-Bil/T-Bil[ace]		0.77	0.72-0.81	0.74	0.59-0.80	0.54	0.40-0.74
AST/ALT[ce]		1.38	0.94-1.97	1.48	1.10-2.66	2.46	1.95-3.63
TBA/D-Bil[ace]		0.85	0.72-1.09	1.04	0.92-1.14	3.36	1.98-4.43
D-Bil/TCH[ac]		30.2	22.8-34.0	21.5	16.7-31.2	18.7	13.9-26.6
TBA/TCH[ce]		24.7	19.8-30.2	24.2	21.4-26.9	60.7	32.4-70.9

[a]P < 0.05 between BA and INC; [c]P < 0.05 between BA and NICCD; [e]P < 0.05 between INC and NICCD. NICCD: Neonatal intrahepatic cholestasis caused by citrin deficiency; BA: Biliary atresia; INC: Idiopathic neonatal cholestasis; TBA: Total bile acid; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyltranspeptidase; TCH: Total cholesterol.

group than in the other two groups. The NICCD group also had significantly higher TBA and lower D-Bil and cholesterol levels compared with the BA and INC groups, indicating that the excretion of bile acids, D-Bil and cholesterol might be affected differently in NICCD patients. Significantly lower T-Bil and GGT levels were noticed in the NICCD group only when compared with the BA group.

Comparison of ratios of biochemical indices

To compare further the different biochemical indices, a series of ratios was calculated. The highest ratio of D-Bil to T-Bil was found in the BA group and the lowest in the NICCD group (Table 2, Figure 1F). A much higher AST/ALT ratio was found in the NICCD group compared to the INC and BA groups (Table 2).

The ratios between D-Bil, bile acids and cholesterol were also compared (Table 2). The ratio of serum TBA to D-Bil was significantly higher in the NICCD group than the ratios in the BA and INC groups (Figure 1G, P < 0.05). Significant differences were also found for the ratio of TBA to TCH between the NICCD and BA groups and between the NICCD and INC groups (Figure 1H, P < 0.05).

DISCUSSION

Citrin deficiency is one of the most common classical inborn errors of metabolism of amino acids, organic acids and fatty acid oxidation in Eastern Asia, including China[24]. The biochemical characteristics and mechanism of cholestasis caused by citrin deficiency are still not fully understood. Although differences in some indices among patients with BA, INC and NICCD have been reported previously, the very small sample sizes of the studies precluded a definite conclusion[4,19,23]. In the present study, the cohorts of NICCD, INC and BA had numbers large enough to test previous findings. Additionally, by comparing the elevation of D-Bil, TBA and cholesterol levels and the ratios of these compounds, it was found that the excretion of bile acids appeared to be more severely affected in NICCD than in BA and INC.

A previous study with a small number of subjects demonstrated that patients with cholestasis caused by citrin deficiency had lower ALT and AST levels and higher AST to ALT ratios compared to those with BA or idiopathic neonatal hepatitis[25]. Low albumin and glucose levels were also associated with NICCD in a previous case series[26,27]. In the present study, those findings were confirmed. Previous studies also showed that patients with NICCD had lower birth weight compared to normal controls or to the national standard. In our study, although a lower median birth weight in the NICCD group was noticed, the differences did not reach statistical significance compared to patients with BA or INC. This could be explained by the different control groups (normal control or national standard used in previous studies *vs* patients with cholestasis of other causes) and the large difference observed within the NICCD group in this study.

The serum TBA level in NICCD has previously been compared with that in BA and INC in a study that had very few subjects[19]. In the present study, the serum level of TBA as well as the ratio of serum TBA to D-Bil and cholesterol levels was compared. In BA, we can suppose that excretion of D-Bil, bile acids and cholesterol is affected to the same extent in consideration of complete blockage of the biliary system. If the ratio of TBA to D-Bil in the BA group was taken as the reference value, the median for INC was found to be 1.22 (1.04/0.85) times higher

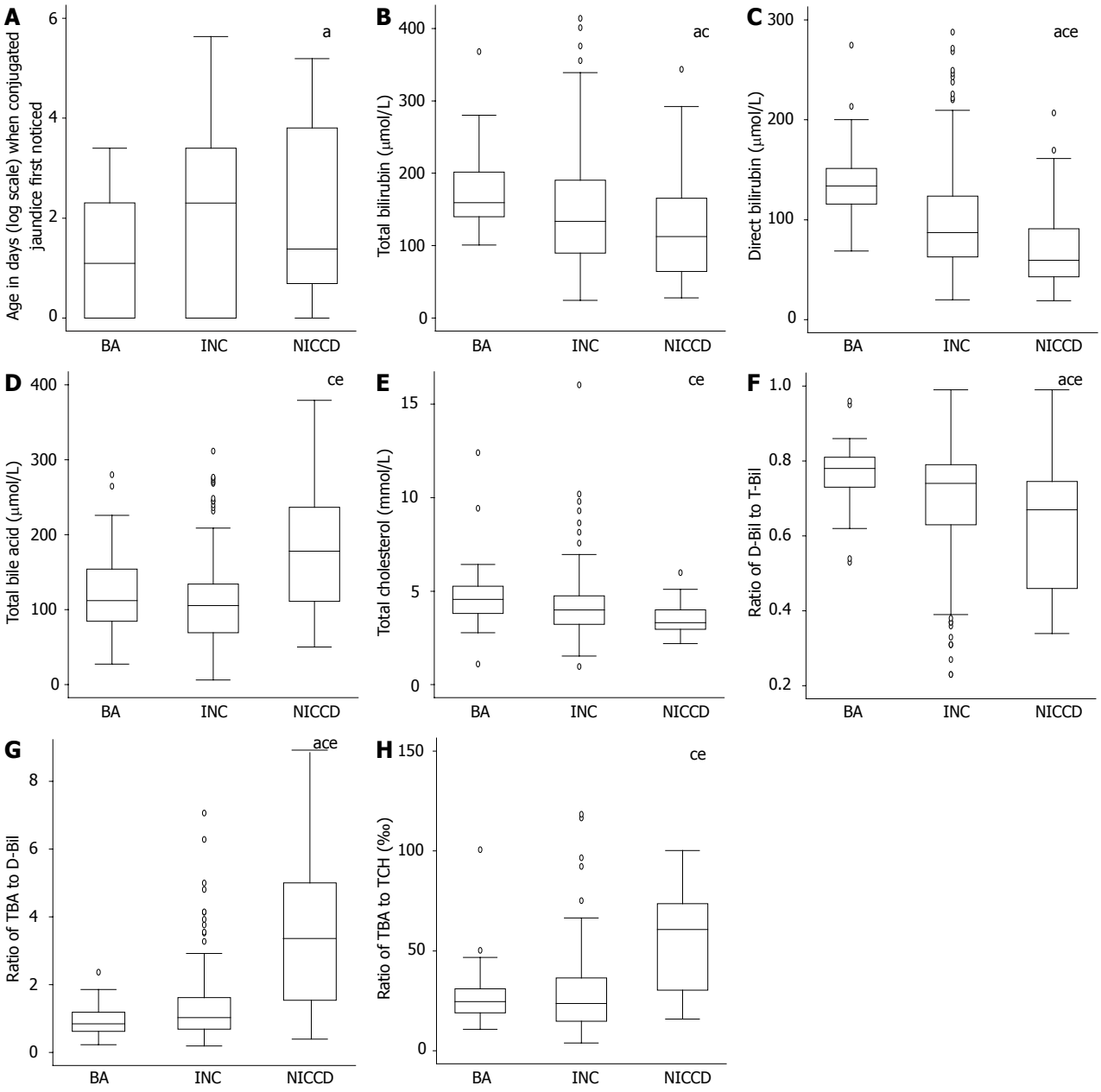


Figure 1 Comparison of the age when conjugated jaundice was first noticed (A), biochemistry indices (B-E), and ratios of some biochemical indices (F-H) in patients with neonatal intrahepatic cholestasis caused by citrin deficiency, idiopathic neonatal cholestasis and biliary atresia. $^{a}P < 0.05$ between BA and INC; $^{c}P < 0.05$ between BA and NICCD; $^{e}P < 0.05$ between INC and NICCD. Normal range: T-Bil (2-20 μmol/L), D-Bil (0-6 μmol/L), total bile acids (< 40 μmol/L), and TCH (3.12-5.20 mmol/L). NICCD: Neonatal intrahepatic cholestasis caused by citrin deficiency; BA: Biliary atresia; INC: Idiopathic neonatal cholestasis; TBA: Total bile acid; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; TCH: Total cholesterol.

and that of NICCD 3.95 (3.36/0.85) times higher. For the ratio of TBA to cholesterol, if the median value of the BA group was taken as a standard, the median in the INC group was nearly the same as the standard but that in the NICCD group was 2.46 (60.7/24.7) times higher. These results indicate that the excretion of bile acids is much more severely affected than the excretion of bilirubin and cholesterol in NICCD patients. As a consequence, we may speculate that the failure to excrete bile acids from hepatocytes to the canalicula is the main mechanism of cholestasis caused by citrin deficiency.

The main limitation of this study was its retrospective nature. It could be argued that some biochemical indices were affected by the drugs that patients were taking. However, prior to the determination of a clear diagnosis, the management of patients had been similar in the three groups; therefore, the patients in the different groups would have been affected by these variables in the same way. Another measure that was used to avoid sample bias was using the first available laboratory data obtained when patients were referred to us. Although significant differences in TBA and TBA ratios were found between the NICCD and other two groups, no cut-off levels can be presented at this time.

COMMENTS

Background

Citrin deficiency is one of the most common metabolic disorders in Eastern Asia. It has at least two main phenotypes: neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and adult-onset type Ⅱ citrullinemia. The clinical features of and the mechanism of cholestasis in NICCD have yet to be established.

Research frontiers

Some biochemical indices of patients with NICCD have been compared to those of patients with biliary atresia (BA) and of patients with idiopathic neonatal cholestasis (INC). Comparison of these biochemical indices in neonatal cholestasis cases with different etiologies will better characterize the biochemical changes of the disease and may further the understanding of the mechanism of cholestasis caused by citrin deficiency.

Innovations and breakthroughs

Apart from confirming previous findings that NICCD patients had significantly lower alanine aminotransferase (ALT) level, lower direct bilirubin (D-Bil) to total bilirubin ratio, and significantly higher aspartate aminotransferase to ALT ratio compared to the BA and INC patients, this study specifically compared the serum level of total bile acid (TBA) and its ratio to D-Bil and cholesterol, and found that NICCD patients had significantly higher TBA levels as well as higher TBA to D-Bil and TBA to cholesterol ratios than patients with BA and INC.

Applications

The excretion of TBA appears to be much more severely disturbed than that of D-Bil and cholesterol in cholestasis caused by citrin deficiency. Further study of this condition will help elucidate the mechanism of cholestasis in NICCD, and the ratios could be further developed as indices for the differential diagnosis of neonatal cholestasis.

Peer review

The authors present an interesting retrospective study comparing liver specific biochemical parameters in different groups of infants with cholestasis.

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BRIEF ARTICLE

Colonic stenting *vs* emergent surgery for acute left-sided malignant colonic obstruction: A systematic review and meta-analysis

Guang-Yao Ye, Zhe Cui, Lu Chen, Ming Zhong

Guang-Yao Ye, Zhe Cui, Lu Chen, Ming Zhong, Department of General Surgery, Renji Hospital affiliated to Shanghai Jiaotong University School of Medicine, Shanghai 200001, China
Author contributions: Ye GY, Cui Z and Chen L designed the study, collected and analyzed the data and wrote the manuscript; Zhong M analyzed the data and contributed to the discussion; Ye GY and Cui Z independently assessed the titles and abstracts of all identified citations; Chen L resolved the disagreement; Ye GY and Cui Z evaluated the full text of all potentially eligible papers and decided whether to include or exclude each study according to the inclusion and exclusion criteria.
Correspondence to: Lu Chen, Professor, MD, Department of General Surgery, Renji Hospital affiliated to Shanghai Jiaotong University School of Medicine, Shanghai 200001, China. chenlu_65@163.com
Telephone: +86-21-53882032 Fax: +86-21-63261383
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Abstract

AIM: To investigate the effects of emergent preoperative self-expandable metallic stent (SEMS) *vs* emergent surgery for acute left-sided malignant colonic obstruction.

METHODS: Two investigators independently searched the MEDLINE, EMBASE and Cochrane Central Register of Controlled Trials, as well as references of included studies to identify randomized controlled trials (RCTs) that compared two or more surgical approaches for acute colonic obstruction. Summary risk ratios (RR) and 95% CI for colonic stenting and emergent surgery were calculated.

RESULTS: Eight studies met the selection criteria, involving 444 patients, of whom 219 underwent SEMS and 225 underwent emergent surgery. Seven studies reported difference of the one-stage stoma rates between the two groups (RR, 0.60; 95% CI: 0.48-0.76; $P < 0.0001$). Only three RCTs described the follow-up stoma rates, which showed no significant difference between the two groups (RR, 0.80; 95% CI: 0.59-1.08; $P = 0.14$). Difference was not significant in the mortality between the two groups (RR, 0.91; 95% CI: 0.50-1.66; $P = 0.77$), but there was significant difference (RR, 0.57; 95% CI: 0.44-0.74; $P < 0.0001$) in the overall morbidity. There were no significant differences between the two groups in the anastomotic leak rate (RR, 0.60; 95% CI: 0.28-1.28; $P = 0.19$), occurrence of abscesses, including peristomal abscess, intraperitoneal abscess and parietal abscess (RR, 0.83; 95% CI: 0.36-1.95; $P = 0.68$), and other abdominal complications (RR: 0.67; 95% CI: 0.40-1.12; $P = 0.13$).

CONCLUSION: SEMS is not obviously more advantageous than emergent surgery for patients with acute left-sided malignant colonic obstruction.

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Key words: Acute obstruction; Colonic cancer; Self-expandable metallic stent; Stoma placement; Meta-analysis; Systematic review

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INTRODUCTION

Colorectal cancer is the fourth most common malignancy worldwide, with an estimated number of 1 023 000 new cases and 529 000 deaths each year[1]. The incidence of colorectal cancer has been increasing rapidly in Asia over the past few decades[2]. About 7%-29% of the patients with colorectal cancer present with bowel obstruction[3,4]. And benefit of surgical management of malignant large bowel obstruction remains controversial, especially for left-sided colonic obstruction. Emergent colorectal surgery for acute obstruction is associated with a mortality rate of 15%-20% and a morbidity rate of 15%-20%, both significantly higher than that in the elective situation[5-7]. Emergent surgery is an independent factor of mortality and morbidity, and about two-thirds of patients end up with a permanent stoma[3,6,8,9].

In 1991, colonic stenting was introduced to restore luminal patency in patients with malignant obstruction of the left side colon[10]. Tejero *et al*[11] used self-expandable metallic stent (SEMS) as a bridge to surgery in two patients with colonic obstruction in 1994. Stent placement before elective surgery, also known as a bridge to surgery, improved the clinical condition of the patient and seemed to decrease the mortality, morbidity, and number of colostomies in uncontrolled studies[12-15]. Although preoperative SEMS insertion has such advantages, it may result in the related complications such as perforation, stent migration, and reobstruction. As shown in the recent randomized controlled trials (RCTs), whether preoperative SEMS can reduce mortality, complication rate and stoma rate is still a big controversy[16-18]. Thus, this meta-analysis was performed to evaluate the effects of preoperative SEMS *vs* emergent surgery for acute left-sided malignant colonic obstruction.

MATERIALS AND METHODS

Search strategy

SEMS was first used in 1991. We therefore, searched the databases, including the Cochrane Central Register of Controlled Trials (1991-April 2011), MEDLINE (1991-September 2011), EMBASE (1991-September 2008), Elsevier ScienceDirect (1998-September 2008), SpringLink (up to September 2011), Ovid LWW (1991-September 2011) and BMJ Journals Online (up to September 2008). The following keywords were used: "intestinal obstruction", "colon", "rectum", "left-sided colon", "surgery", "resection", "stents", "randomized" and "controlled study". The detailed search strategy is available from the authors. All included studies also had access to the PubMed "related articles" function and the Science Citation Index. In addition, the reference lists of included studies were scrutinized. No language restrictions were applied.

Data extraction

Data were independently abstracted from each study by two researchers, and disagreement was resolved by consensus. Data were extracted from each study using a pre-designed review form. Data to be extracted were as follows: (1) treatment details: primary anastomosis rate, and the incidence of stoma creation; (2) short-term adverse events: mortality and morbidity such as anastomotic leak rate, abscess and extra abdominal complications; and (3) long-term outcomes: follow-up stoma rate.

Inclusion and exclusion criteria

Studies fulfilling the following criteria were included in the meta-analysis: (1) RCTs or other comparative studies comparing SEMS as a bridge to surgery and emergent surgery; (2) reports on at least one of the outcome measures mentioned below; and (3) studies reporting patients with malignant acute left-sided colonic obstruction.

Quality of methodology

The quality of nonrandomized studies was assessed using the Newcastle-Ottawa Scale with some modifications to meet the needs for this meta-analysis[19], and the quality of randomized studies was evaluated by means of the modified Jadad score[20]. The quality of the studies was evaluated based on three items: patient selection, comparability of study groups, and assessment of outcome. Studies achieving five or more stars were considered high quality. The quality of randomized studies was evaluated by means of the modified Jadad score including the following four areas: (1) randomization method; (2) hidden subgroups; (3) blinding; and (4) the description of the loss to follow-up and drop-out and the intention-to-treat. The total score of 1 to 3 points were ascribed to low-quality studies, whereas a total score of 4 to 7 points to high-quality researches.

Statistical analysis

Using the Cochrane Collaboration's RevMan 5.1 software provided by meta-analysis, the results of included measurement of indicators were all count data, and 95% CI was used for the efficacy analysis. The heterogeneity between studies was tested. When there was homogeneity among studies ($P > 0.1$, $I^2 < 50\%$), a fixed effects model was used for meta-analysis; if there is significant heterogeneity among studies ($P < 0.1$, $I^2 > 50\%$), the random effects model was used. We also analyzed the different quality of the possible causes, and conducted subgroup analysis. If the heterogeneity among the studies was too large, descriptive analysis was performed.

RESULTS

Selection of trials

The initial search strategy retrieved 88 articles after screening all titles, abstracts and full texts. Twenty-two articles were excluded due to lack of comparison with other surgical strategies in most of the cohort studies, 45 articles were excluded because of comparison stenting *vs* surgery without a bridge to the surgery, and 13 studies were excluded because there was no control. Finally, 8 trials with 444 patients were included, of whom 219

Table 1 Randomized controlled trials involved in the meta-analysis

Year	Author	Region	Total	SEMS	Surgery	Concealment of allocation	Jaded score	Quality
2009	Cheung	Hongkong	60	30	30	Appropriant	5	High
2010	Pirlet	France	48	24	24	Appropriant	5	High
2011	Van Hooft	Holland	98	47	51	Appropriant	5	High

SEMS: Self-expandable metallic stent.

Table 2 Basic characteristics of included nonrandomized controlled studies in the meta-analysis

Year	Author	Design	Total	SEMS	Surgery	Match	Study quality (rate, max 11)
2008	Dastur	R	43	19	23	1,2,3,4,5,6	8
2002	Martin	R	52	26	26	1,2,3,4,5,8	7
2006	Ng	R	60	20	40	1,2,3,5,6,8	8
2007	Pessione	R	16	9	7	1,2,3,4,5,8	7

Matching: 1 = age, 2 = sex, 3 = diagnosis, 4 = tumor site, 5 = tumor stage, 6 = American Society of Anesthesiologists score, 7 = body mass index, 8 = comorbidity. SEMS: Self-expandable metallic stent. "R" represents that the study was a randomized clinical trail.

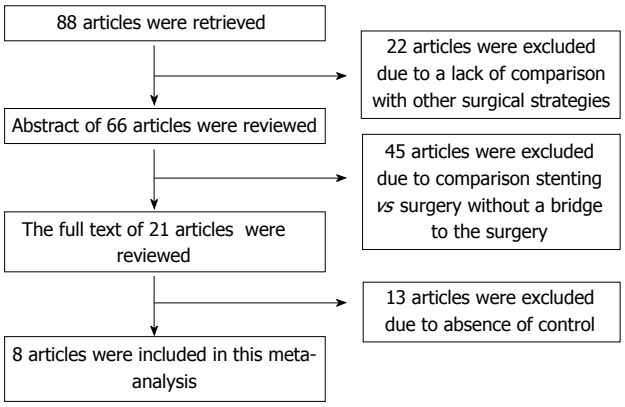


Figure 1 Flow chart of selection of studies and reasons for exclusion from the meta-analysis.

(49.3%) successfully underwent stent insertion and 225 (50.7%) underwent emergent surgery. There were 17 (7.8%) deaths in the SEMS as a bridge to surgery group and 21 (9.3%) deaths in the emergent surgery group. There were only three RCTs[16-18] and five nonrandomized controlled studies (NRCTs)[12-14,21,22]. The flow chart of selection of studies and reasons for exclusion is presented in Figure 1. Characteristics of studies included in the meta-analysis are presented in Tables 1 and 2.

Quality of studies

Three RCTs were of moderate to good methodological quality evaluated by the modified Jadad score[20]. Because of the special strategies under assessment, no blind method was used in all the RCTs. All five NRCTs contained groups matched for age, sex, and diagnosis; five articles contained information on tumor site, tumor stage, American Society of Anesthesiologists score, or body mass index, respectively. All studies were scored more than five stars using the modified Newcastle-Ottawa scale[19].

Meta-analysis of treatment details and long-term outcomes

Our meta-analysis showed statistically significant difference between the SEMS group (175 patients) and the emergent surgery group (201 patients) in seven studies with regard to the one-stage stoma rate [risk ratios (RR): 0.60, 95% CI: 0.48-0.76; $P < 0.0001$]. There was no significant heterogeneity between the studies ($P = 0.14$, $I^2 = 37\%$) (Figure 2A). Only three RCTs compared the two groups (101 patients in the SEMS group and 105 in the emergent surgery group) and described the follow-up stoma rates. There was neither significant difference in follow-up stoma rates (RR: 0.80; 95% CI: 0.59-1.08; $P = 0.14$), nor heterogeneity ($P = 0.17$, $I^2 = 44\%$) between the two groups (Figure 2B).

Anastomosis rates were reported by all eight trials involving 219 patients in the SEMS group and 225 in the emergent surgery group. There was a significant difference between the two groups, with a pooled RR of 1.64 (95% CI: 1.39-1.94; $P < 0.00001$) (Figure 2C).

Meta-analysis of short-term adverse events: Mortality and morbidity such as anastomotic leak rate, abscess and extra abdominal complications

Six trials reported mortality in 149 patients in the SEMS group and 175 patients in the emergent surgery group. There was no significant difference (RR: 0.91; 95% CI: 0.50-1.66; $P = 0.77$) and heterogeneity ($P = 0.65$, $I^2 = 0\%$) between the two groups (Figure 2D).

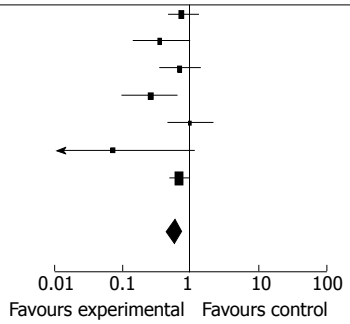
Seven trials with 200 patients in the SEMS group and 218 patients in the emergent surgery group reported the overall morbidity. There was no significant difference between the two groups with a pooled RR of 0.57(95% CI; 0.44-0.74; $P < 0.0001$). However, significant heterogeneity was observed ($P = 0.0001$, $I^2 = 78\%$) (Figure 2E).

Further complication analysis was also performed, such as anastomotic leak, abscess and extra abdominal complications. The incidence of anastomotic leakage in

A

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Risk ratio M-H, fixed, 95% CI
Pirlet 2010	13	30	17	30	16.1%	0.76 [0.46, 1.28]
Cheung 2009	4	24	11	24	10.4%	0.36 [0.13, 0.98]
Dastur 2008	7	19	12	23	10.3%	0.71 [0.35, 1.43]
Martinez-Santos 2002	4	26	15	26	14.2%	0.27 [0.10, 0.70]
Ng 2006	7	20	14	40	8.8%	1.00 [0.48, 2.08]
Pessione 2007	0	9	5	7	5.8%	0.07 [0.00, 1.13]
van Hooft 2011	24	47	38	51	34.5%	0.69 [0.50, 0.95]
Total (95% CI)		175		201	100.0%	0.60 [0.48, 0.76]
Total events	59		112			

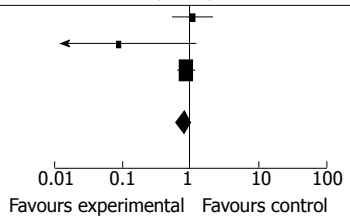
Heterogeneity: Chi^2 = 9.58, df = 6 (P = 0.14); I^2 = 37%
Test for overall effect: Z = 4.32 (P < 0.0001)



B

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Risk ratio M-H, fixed, 95% CI
Pirlet 2010	9	30	8	30	17.0%	1.13 [0.50, 2.52]
Cheung 2009	0	24	6	24	13.8%	0.08 [0.00, 1.29]
van Hooft 2011	27	47	34	51	69.2%	0.86 [0.63, 1.18]
Total (95% CI)		101		105	100.0%	0.80 [0.59, 1.08]
Total events	36		48			

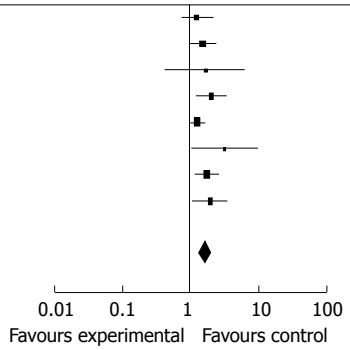
Heterogeneity: Chi^2 = 3.56, df = 2 (P = 0.17); I^2 = 44%
Test for overall effect: Z = 1.46 (P = 0.14)



C

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Risk ratio M-H, fixed, 95% CI
Pirlet 2010	18	30	14	30	14.8%	1.29 [0.79, 2.08]
Cheung 2009	20	24	13	24	13.8%	1.54 [1.02, 2.32]
Dastur 2008	9	19	2	7	3.1%	1.66 [0.47, 5.87]
Martinez-Santos 2002	22	26	11	26	11.7%	2.00 [1.24, 3.23]
Ng 2006	19	20	29	40	20.5%	1.31 [1.06, 1.63]
Pessione 2007	9	9	2	7	2.9%	3.04 [1.08, 8.58]
Saida 2003	34	44	18	40	20.0%	1.72 [1.18, 2.51]
van Hooft 2011	23	47	13	51	13.2%	1.92 [1.10, 3.34]
Total (95% CI)		219		225	100.0%	1.64 [1.39, 1.94]
Total events	154		102			

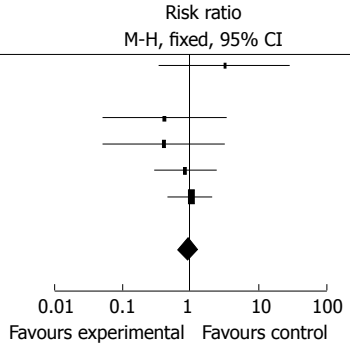
Heterogeneity: Chi^2 = 7.66, df = 7 (P = 0.36); I^2 = 9%
Test for overall effect: Z = 5.83 (P < 0.00001)



D

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Risk ratio M-H, fixed, 95% CI
Pirlet 2010	3	30	1	30	5.2%	3.00 [0.33, 27.23]
Cheung 2009	0	24	0	24		Not estimable
Dastur 2008	1	19	3	23	14.2%	0.40 [0.05, 3.57]
Ng 2006	1	20	5	40	17.5%	0.40 [0.05, 3.20]
Pessione 2007	3	9	3	7	17.7%	0.78 [0.22, 2.74]
van Hooft 2011	9	47	9	51	45.3%	1.09 [0.47, 2.50]
Total (95% CI)		149		175	100.0%	0.91 [0.50, 1.66]
Total events	17		21			

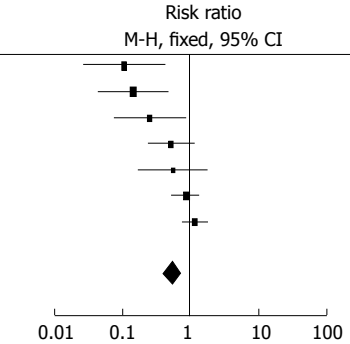
Heterogeneity: Chi^2 = 2.49, df = 4 (P = 0.65); I^2 = 0%
Test for overall effect: Z = 0.29 (P = 0.77)



E

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Risk ratio M-H, fixed, 95% CI
Cheung 2009	2	24	17	24	16.3%	0.12 [0.03, 0.45]
Saida 2003	3	44	17	40	17.1%	0.16 [0.05, 0.51]
Martinez-Santos 2002	3	26	11	26	10.6%	0.27 [0.09, 0.87]
Ng 2006	6	20	22	40	14.1%	0.55 [0.26, 1.13]
Pessione 2007	3	9	4	7	4.3%	0.58 [0.19, 1.80]
Pirlet 2010	15	30	17	30	16.3%	0.88 [0.55, 1.42]
van Hooft 2011	25	47	23	51	21.2%	1.18 [0.79, 1.77]
Total (95% CI)		200		218	100.0%	0.57 [0.44, 0.74]
Total events	57		111			

Heterogeneity: Chi^2 = 27.04, df = 6 (P = 0.0001); I^2 = 78%
Test for overall effect: Z = 4.30 (P < 0.0001)



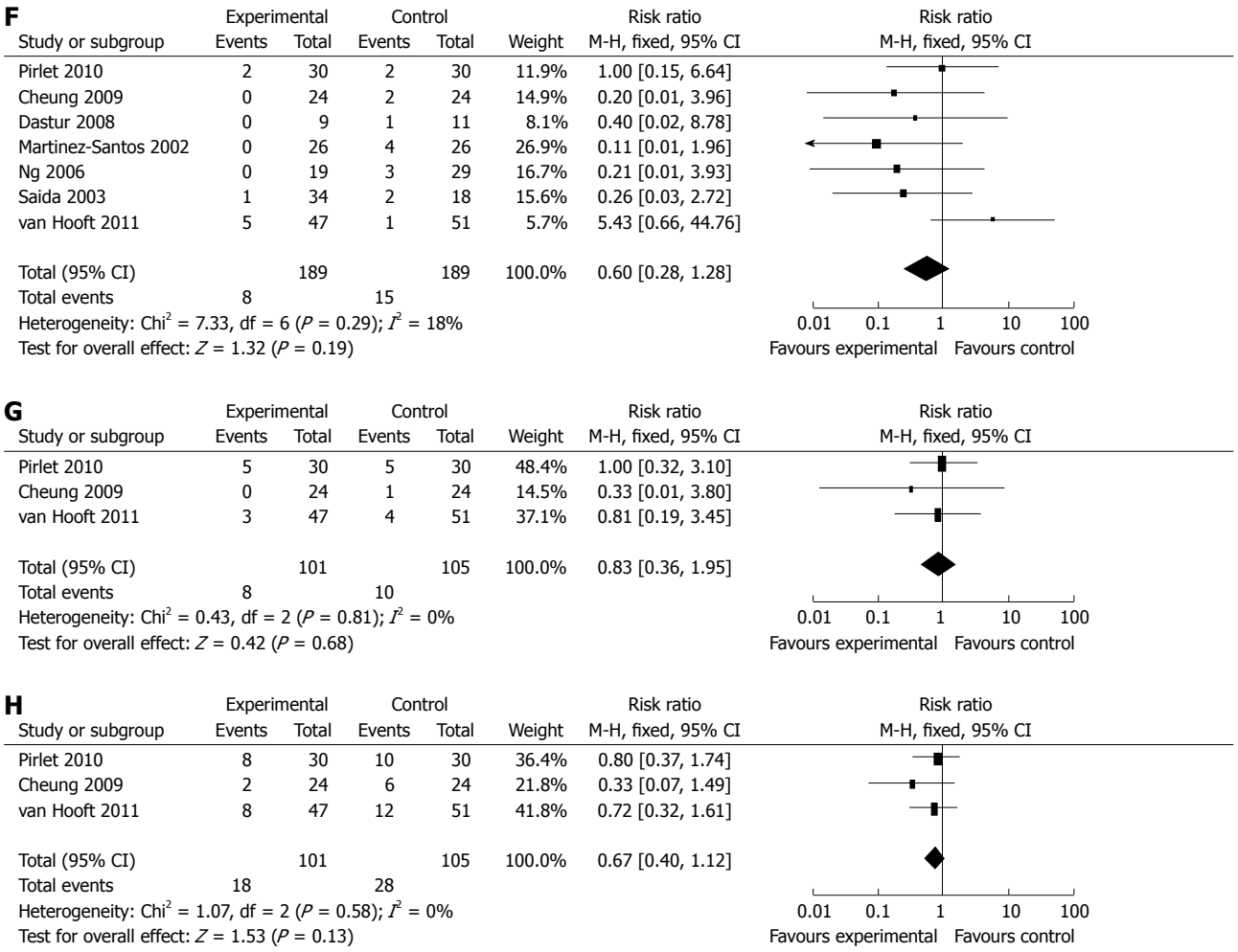


Figure 2 Colonic stenting *vs* emergent surgery. A: One-stage stoma rates; B: Follow-up stoma rates; C: Anastomosis rates; D: Mortality; E: Morbidity; F: Anastomotic leak; G: Abscesses; H: Extra abdominal complication. M-H: Mantel Haenszel.

the two groups was reported in seven studies. SEMS did not reduce the incidence of anastomotic leakage in patients treated with emergent surgery (RR: 0.60; 95% CI: 0.28-1.28; $P = 0.19$). No significant heterogeneity was observed between the two groups ($P = 0.29$, $I^2 = 18\%$) (Figure 2F).

Three studies compared the rates of abscesses, including peristomal abscess, intraperitoneal abscess and parietal abscess and extra abdominal complication. The analyses showed no significant difference in occurrence of abscess (RR: 0.83, 95% CI: 0.36-1.95; $P = 0.68$) and extra abdominal complications (RR: 0.67, 95% CI: 0.40-1.12; $P = 0.13$). No significant heterogeneity was found in abscess ($P = 0.81$, $I^2 = 0\%$) (Figure 2G) and extra abdominal complications ($P = 0.58$, $I^2 = 0\%$) (Figure 2H).

RCTs vs NRCTs

We also compared three RCTs with other NRCTs. There were significant differences in the morbidity (RCT group, RR 1.18; 95% CI: 0.79-1.77; $P = 0.08$ *vs* NRCTs group, RR 0.34; 95% CI: 0.21-0.56; $P < 0.0001$), anastomotic leak rate (RCT group, RR 5.43; 95% CI: 0.66-44.76; $P = 0.53$, *vs* NRCT group, RR 0.21; 95% CI: 0.05-0.82; $P = 0.02$), and anastomosis rates (RCT group, RR 1.57; 95% CI: 1.10-2.08; $P = 0.002$, *vs* NRCT group, RR 1.69; 95% CI: 1.38-2.08; $P < 0.00001$). There were no differences in the stoma rates (RCT group, RR 0.65; 95% CI: 0.50-0.85; $P = 0.002$, *vs* NRCT group, RR 0.52; 95% CI: 0.34-0.08; $P = 0.003$) and mortality (RCT group, RR 1.09; 95% CI: 0.47-2.50; P=0.53, *vs* NRCT group, RR 0.54; 95% CI: 0.2-1.45; $P = 0.22$).

DISCUSSION

Meta-analysis can be used to evaluate the existing literature both qualitatively and quantitatively by comparing and integrating the results of different studies and taking into account the variations in characteristics that could influence the overall estimate of the outcome of interest[23]. Although meta-analysis is traditionally applied and best confined to RCTs, meta-analytical techniques using NRCTs might be a good method in some clinical settings in which either the number or the sample size of RCTs was insufficient[24].

The concept of colonic stenting as a bridge to elective surgery in patients with acute left-sided malignant

colonic obstruction has been established to reduce the morbidity, mortality and number of colostomies. The nonrandomized or retrospective studies showed a significant reduction of morbidity and mortality, and need for stoma placement when SEMS was inserted before surgery with palliative intent. In contrast to these studies, two RCTs failed to confirm the findings.

Our meta-analysis illustrated that SEMS placement significantly decreased the one-stage stoma rates (RR: 0.60; 95% CI: 0.48-0.76; $P < 0.0001$) and increased anastomosis rates (RR: 1.64; 95% CI: 1.39-1.94; $P < 0.00001$), but no difference was found by the end of follow-up. The results of 206 patients in three RCTs which reported follow-up stoma rates demonstrated no difference. The difference of the stoma rate in the follow-up was partly caused by the high leakage rate of primary anastomosis in one stent group[16], probably because bowel decompression and improvement of the patients' clinical condition were insignificant at the time of elective operation. Another reason might be that more patients with complete obstruction had been selected in a Holland study[16]. In a retrospective study from a renowned tertiary referral centre, complete obstruction has been identified as a risk factor for complications[25]. Additionally, the elective nature of the operation and the surgeons' faith in the idea of bridge to surgery might have made the surgeons less conservative than the emergent surgery group.

The perioperative mortality is frequently used to evaluate the outcome of SEMS. Our study failed to reveal the difference between the two groups. Six studies including 324 patients came to a conclusion that there was no difference (RR: 0.91; 95% CI: 0.50-1.66; $P = 0.77$) between SEMS group and emergent surgery group. These outcomes might imply the potential benefits for preoperative SEMS as a bridge to surgery. It is hard to evaluate this outcome as the patients picked up did not match with the clinical stages. Clinical stage is considered as one of independent factors for prognosis. When SEMS is used as a bridge to surgery, there is concern about the oncologic outcome of those patients whose disease is potentially curable, because theoretically SEMS placement could induce tumor dissemination and worsen long-term survival[26]. More subgroup analyses should be performed to obtain a more accurate assessment.

In acute colonic obstruction, doctors and patients both want to remove the tumor with primary anastomosis with a shorter hospital stay. But the major concern is how to avoid complications. In right-sided bowel obstruction, a resection with primary anastomosis has been generally accepted by surgeons, but it is controversial in left-sided bowel obstruction. It is believed that the left colon obstruction has a high risk of radical resection and anastomosis with a high incidence of complications. Even with modern enema techniques and nutritional support, the rates of postoperative complications are still as high as 40%-50% (both significantly higher than in the elective situation with a < 14.0% anastomotic leakage rate and 10.0% operative mortality)[5,6,15,17,21-23,27,28] because of the long procedure time, peritoneal contamination, thin proximal wall of obstruction bowel, inflammatory edema, and poor blood supply. Although preoperative SEMS can potentially ameliorate bowel edema, there was no improvement in the overall situation. There was a significant difference between the two groups in the overall complications in seven studies with 418 patients, (RR: 0.57; 95% CI: 0.44-0.74; $P < 0.0001$). We found that SEMS, as a bridge to elective surgery, could decrease the incidence of anastomotic leakage. Leakage occurred in eight patients in the stenting group as compared with 15 patients in the control group. Anastomotic leakage could increase local recurrence and postoperative mortality. SEMS as a bridge to surgery can provide abundant bowel preparation to decrease tissue edema. But, there was no difference in the operation-related complications as shown in three studies involving 206 patients with abscess (RR: 0.83; 95% CI: 0.36-1.95; $P = 0.68$) and extra abdominal complications (RR: 0.67; 95% CI: 0.4-1.12; $P = 0.13$). Abscess was found as the main complication and pneumonia as the most common adverse event.

The major adverse events occurring in the SEMS group was bowel perforation during the stent placement procedure. Procedure- and stent-related complications were found in 5%-23.1% of patients, with an average rate of stent-related perforations of 5%[29,30]. The oncological consequences of potential tumor dissemination caused by perforations are unclear[15]. The data from NRCTs are inconsistent, ranging from no difference between colonic stenting and emergent surgery to a significantly reduced 5-year survival rate for patients treated with colonic stenting before elective surgery[27]. But the possibility of dissemination should be taken into account and the silent perforations should not be disregarded.

A cost-effectiveness analysis, including cost per quality-adjusted life-year as an outcome measure, was also performed in the meta-analysis. As a result, mortality, morbidity, quality-of-life dimensions, and stoma rates between treatment groups suggest that the probability of colonic stenting which could become more effective than emergent surgery is negligible. As only two authors have evaluated the cost-effectiveness, the results may have limitations, and a large sample would be gathered and assessed.

As this meta-analysis has a few limitations, there might be bias in the results. First, only three studies were RCTs, and confounding factors such as age and gender inevitably existed. Second, there was difference in the selection criteria, such as different protocols, method of procedures and so on. Finally, publication bias might exist when the meta-analysis was based on published studies, because positive results are more likely to be published than negative results.

In summary, the current meta-analysis demonstrated that SEMS as a bridge to surgery for obstructed left-sided colon cancer decreased the incidence of primary stoma rates and anastomotic leakage. But the consequence

failed to show the effect on mortality and complications related to surgery. Therefore, preoperative SEMS can be used as an alternative approach for emergent surgery, but should be used with caution, mainly because of concerns of overt and silent perforations. Future studies are needed to further investigate the oncological outcomes and establish whether specific groups of patients could benefit more from either colonic stenting or emergent surgery.

COMMENTS

Background

Colorectal cancer is one of the most commonly diagnosed malignancies worldwide. And many patients with colorectal cancer present with an acute left-sided colonic obstruction. The benefit of surgical management of malignant large bowel obstruction remains controversial. Stent placement before elective surgery as a bridge to surgery is an alternative for emergent surgery in patients with acute left-sided malignant colonic obstruction. Its benefits are uncertain.

Research frontiers

The authors performed a systematic review of the literature and meta-analysis of the one-stage stoma rates, follow-up stoma rates, anastomotic leakage rates, abscess, extra abdominal complications, morbidity and mortality of SEMS compared with emergent surgery.

Innovations and breakthroughs

The current meta-analysis demonstrated the advantage of SEMS as a bridge to surgery for obstructed left-sided colon cancer in term of decreasing the incidence of primary stoma rates and anastomotic leakage. But the consequence failed to show the effect on mortality and complications related to surgery.

Applications

The analysis has shown that preoperative SEMS can be used as an alternative approach for emergent surgery, but should be used with caution, mainly because of the concerns of overt and silent perforations. Future studies are needed to further investigate oncological outcomes and establish whether specific groups of patients could benefit more from either colonic stenting or emergent surgery.

Terminology

SEMS: SEMS is a metallic tube or stent, used to hold a structure in the gastrointestinal tract in order to allow the passage of food, stool, or other secretions required for digestion; Systematic review: A literature review focused on a research question that tries to identify, appraise, select and synthesize all high quality research evidence relevant to that question; Meta-analysis: A combination of the results of several studies that address a set of related research hypotheses.

Peer review

Overall, this is a nice review with good metrics and appropriate analysis.

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BRIEF ARTICLE

Comparison between different reconstruction routes in esophageal squamous cell carcinoma

Yu-Zhen Zheng, Shu-Qin Dai, Wei Li, Xun Cao, Xin Wang, Jian-Hua Fu, Peng Lin, Lan-Jun Zhang, Bin Lu, Jun-Ye Wang

Yu-Zhen Zheng, Xun Cao, Xin Wang, Jian-Hua Fu, Peng Lin, Lan-Jun Zhang, Bin Lu, Jun-Ye Wang, Department of Thoracic Oncology, Sun Yat-Sen University Cancer Center, Guangzhou 510060, Guangdong Province, China
Shu-Qin Dai, Department of Clinical Laboratory, Sun Yat-Sen University Cancer Center, Guangzhou 510060, Guangdong Province, China
Wei Li, Department of Anesthesia, Sun Yat-Sen University Cancer Center, Guangzhou 510060, Guangdong Province, China
Author contributions: Zheng YZ and Dai SQ contributed equally to this work; Zheng YZ and Dai SQ drafted the article; Li W and Cao X revised the manuscript; Wang X, Fu JH and Lin P provided the raw data; Zhang LJ and Lu B analyzed the data; and Wang JY designed the study.
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Correspondence to: Jun-Ye Wang, Professor, Department of Thoracic Oncology, Cancer Center, Sun Yat-Sen University, No.651, Dongfeng Road East, Guangzhou 510060, Guangdong Province, China. zhengyzh@sysucc.org.cn
Telephone: +86-20-87343315 Fax: +86-20-87343268
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Abstract

AIM: To compare postoperative complications and prognosis of esophageal squamous cell carcinoma patients treated with different routes of reconstruction.

METHODS: After obtaining approval from the Medical Ethics Committee of the Sun Yat-Sen University Cancer Center, we retrospectively reviewed data from 306 consecutive patients with histologically diagnosed esophageal squamous cell carcinoma who were treated between 2001 and 2011. All patients underwent radical McKeown-type esophagectomy with at least two-field lymphadenectomy. Regular follow-up was performed in our outpatient department. Postoperative complications and long-term survival were analyzed by treatment modality, baseline patient characteristics, and operative procedure. Data from patients treated *via* the retrosternal and posterior mediastinal routes were compared.

RESULTS: The posterior mediastinal and retrosternal reconstruction routes were employed in 120 and 186 patients, respectively. Pulmonary complications were the most common complications experienced during the postoperative period (46.1% of all patients; 141/306). Compared to the retrosternal route, the posterior mediastinal reconstruction route was associated with a lower incidence of anastomotic stricture (15.8% *vs* 27.4%, $P = 0.018$) and less surgical bleeding (242.8 ± 114.2 mL *vs* 308.2 ± 168.4 mL, $P < 0.001$). The median survival time was 26.8 mo (range: 1.6-116.1 mo). Upon uni/multivariate analysis, a lower preoperative albumin level ($P = 0.009$) and a more advanced pathological stage (pT; $P = 0.006$; pN; $P < 0.001$) were identified as independent factors predicting poor prognosis. The reconstruction route did not influence prognosis ($P = 0.477$).

CONCLUSION: The posterior mediastinal route of reconstruction reduces incidence of postoperative complications but does not affect survival. This route is recommended for resectable esophageal squamous cell carcinoma.

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Key words: Esophageal carcinoma; Route of reconstruction; Posterior mediastinal; Retrosternal; Comparison

Peer reviewer: Satoru Motoyama, MD, PhD, Department of Surgery, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan

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INTRODUCTION

The therapeutic treatment of esophageal cancer has undergone important changes over the past several decades. For patients with localized esophageal cancer, subtotal esophagectomy with a thoracic-abdominal-cervical incision (McKeown-type esophagectomy), combined with extensive lymphadenectomy, is now generally recognized as the optimal treatment in terms of long-term survival[1-5].

After subtotal esophagectomy, a gastric tube formed by resection of the lesser curvature is generally considered to be the most suitable esophageal substitute available[6-10]. Reconstruction under such circumstances commonly uses either the posterior mediastinal (PM) or retrosternal (RS) route[6,10,11]. However, the optimal route remains controversial, principally because most previous studies focused exclusively on postoperative complications and quality of life, rather than prognosis[6-9,12].

Thus, for the first time, we conducted a retrospective study to compare not only the incidence of postoperative complications but also prognosis in patients with esophageal squamous cell carcinoma (ESCC) treated *via* the PM and RS routes.

MATERIALS AND METHODS

Patient selection

After approval was obtained from the Medical Ethics Committee of Sun Yat-Sen University Cancer Center, 306 patients diagnosed with ESCC were consecutively recruited between January 2001 and March 2011. Careful preoperative evaluations were conducted to ensure that there were no contraindications to surgical treatment. All patients included in the present evaluation underwent radical esophagectomy. Exclusion criteria included any history of malignant disease, the presence of a second primary tumor, prior non-curative resection (R1/R2), prior use of an esophageal substitute that was not a gastric tube, and any prior neoadjuvant treatment. Disease was staged based on the recommendations of the American Joint Committee on Cancer (AJCC 2010). The baseline characteristics of the 306 patients enrolled in the present study are shown in Table 1.

Surgery and complications

All patients underwent McKeown-type esophagectomy with at least two-field lymphadenectomy, as described in previous studies[1,4,5]. Three-field lymphadenectomy was performed only if the cervical lymph nodes were thought to be abnormal upon preoperative evaluation. No definitive criteria have been established, therefore, each reconstructive option was determined by the individual surgeon. Pyloroplasty was performed if a patient showed abnormal gastric motility preoperatively. Neither a manubrium nor a partial clavicle was reconstructed.

Complications that occurred during hospital stay and during long-term follow-up were recorded. These included anastomotic leakage, chylothorax, pulmonary complications, cardiac complications, and recurrent laryngeal nerve (RLN) palsy. Drainage, conservative management, symptomatic and function-supportive treatment, and observation, respectively, were used as initial treatments. The treatment of choice for anastomotic stricture (the options included bougienage and stent placement) depended on the extent and history of the stricture. Surgical details, and complications, are summarized in Table 2.

Follow-up

After completion of primary treatment, patients were followed up in our outpatient department every 4-6 mo for the first 3 years and every 12 mo thereafter. Radio-

Table 1 Baseline patient characteristics

Characteristic	Data	Route of reconstruction (%)	
		Posterior mediastinal	Retrosternal
Age (yr)			
Median	58.3	58.6	58.2
Range	32-79	32-79	34-78
≤ 65[1]	233	90 (75.0)	143 (76.9)
> 65	73	30 (25.0)	43 (23.1)
Sex			
Male	237	95 (79.2)	142 (76.3)
Female	69	25 (20.8)	44 (23.7)
BMI (kg/m^2)			
Median	22.3	22.2	22.3
Range	15.2-35.9	15.2-33.8	15.2-35.9
Smoking index			
Median	444.2	483.6	418.7
Range	0-3330	0-3330	0-2000
≤ 400[2]	174	67 (55.8)	107 (57.5)
> 400	132	53 (44.2)	79 (42.5)
Tumor location			
Upper thorax	46	8 (6.7)	38 (20.4)
Middle thorax	153	54 (45.0)	99 (53.2)
Lower thorax	107	58 (48.3)	49 (26.3)
pT status (UICC 7th)			
pT1	30	11 (9.2)	19 (10.2)
pT2	55	26 (21.7)	29 (15.6)
pT3	221	83 (69.2)	138 (74.2)
pN status (UICC 7th)			
pN0	139	58 (48.3)	81 (43.5)
pN1	93	36 (30.0)	57 (30.6)
pN2	53	16 (13.3)	37 (19.9)
pN3	21	10 (8.3)	11 (5.9)
Tumor grade (UICC 7th)			
G1	90	41 (34.2)	49 (26.3)
G2	168	59 (49.2)	109 (58.6)
G3	48	20 (16.7)	28 (15.1)

[1]This signals the commencement of "older age" as defined by the World Health Organization (WHO); [2]Risk stratification for lung cancer as defined by the WHO. BMI: Body mass index; G: Grade; pN status: Pathological node status; pT status: Pathological tumor status; UICC: International Union Against Cancer.

Table 2 Complications arising when either route of reconstruction was used

Variable	Route of reconstruction (%)		*P* value
	PM	RS	
No. of patients	120	186	
Lymphadenectomy			
Three-field	29 (24.2)	32 (17.2)	
Two-field	91 (75.8)	154 (82.8)	0.137
Pyloroplasty	5 (4.2)	9 (4.8)	0.784
Anastomosis			
Left neck	110 (91.7)	176 (94.6)	
Right neck	8 (6.7)	6 (3.2)	
Intrathoracic	2 (1.7)	4 (2.2)	0.360
Thoracic duct			
Reserved	27 (22.5)	80 (43.0)	
Ligation	80 (66.7)	104 (55.9)	
Resected	13 (10.8)	2 (1.1)	< 0.001
Surgical bleeding (mL)	242.8 ± 114.2	308.2 ± 168.4	< 0.001
Complications (short-term)			
Anastomotic leakage	15 (12.5)	23 (12.4)	
Cervical	13 (10.8)	19 (10.2)	
Intrathoracic	1 (0.8)	2 (1.1)	
Mediastinal	0 (0)	2 (1.1)	
Esophagotracheal	1 (0.8)	0 (0)	0.573
Chylothorax	2 (1.7)	17 (9.1)	0.008
Pulmonary	50 (41.7)	91 (48.9)	0.214
Cardiac	16 (13.3)	20 (10.8)	0.494
RLN palsy	22 (18.3)	31 (16.7)	0.707
Complications (long-term)			
Anastomotic stricture	19 (15.8)	51 (27.4)	0.018

PM: Posterior mediastinal; RS: Retrosternal.

therapy and/or chemotherapy and/or surgical resection were adopted if and when local recurrence and/or metastasis occurred. The chosen treatment modality was determined by consideration of symptoms, the physical condition of the patient, and the clinical stage of disease. Patient-specific therapeutic schedules used the best available remedies at any time. Survival status was explored *via* direct telecommunication with patients or family members in October 2011.

Statistical analysis

We used SPSS 19.0 (SPSS, Inc., Chicago, IL) for statistical analysis. Overall survival (OS) was defined as the interval from the date of surgery to the date of death or final clinical follow-up. Correlations between the reconstructive route and clinicopathological characteristics and postoperative complications were assessed using the *t* and χ^2 tests. To detect factors associated with an increased risk of chylothorax, crude and adjusted analyses were performed using both univariate and multivariate logistic regression. Survival was analyzed *via* the Kaplan-Meier method and the differences between curves were assessed with the aid of the log-rank test. Multivariate Cox's regression analysis was performed with inclusion of parameters that prior univariate analysis had identified as significant. $P < 0.05$ was considered to be significant.

RESULTS

Of the 306 patients, 120 and 186 were treated *via* PM and RS reconstruction, respectively. Baseline patient characteristics are summarized in Table 1.

Table 3 Association between recurrent laryngeal nerve palsy, lymphadenectomy, and development of an anastomotic fistula

Variable	Recurrent laryngeal nerve palsy (%)	*P* value
Lymphadenectomy		
Three-field	15 (24.6)	
Two-field	38 (12.4)	0.094
Anastomotic fistula		
No	42 (15.6)	
Yes	11 (28.9)	0.043

Pulmonary complications were the most common during the perioperative period (46.1% of all patients; 141/306). Patients in the RS group were more likely to preserve the thoracic duct ($P < 0.001$). The mean blood loss was 282.6 mL for the entire cohort and was 65.4 mL greater in the RS group than in the PM group ($P < 0.001$). A positive association between development of chylothorax and use of the RS route was observed ($P = 0.008$). Anastomotic stricture, the combined incidence of which was 12.4% (38/306), was more common in the RS than in the PM group ($P = 0.018$). Details of the operations and complications are listed in Table 2. Upon subgroup analysis, RLN palsy was found to be highly associated with three-field lymphadenectomy ($P = 0.094$) and anastomotic fistula ($P = 0.043$) (Table 3).

The median follow-up interval was 32.1 mo for surviving patients. A total of 201 patients were alive at last follow-up. The predicted 1-, 3- and 5-year overall survival rates after primary surgery were 75%, 60% and 50% respectively. The median survival time was 26.8 mo (range: 1.6-116.1 mo).

Preoperative albumin level ($P = 0.009$), pT status ($P = 0.006$), and pN status ($P < 0.001$) were independent factors prognostic for survival upon uni/multivariate analysis (Table 4). However, reconstruction route was not a significant prognostic factor ($P = 0.477$; Figure 1).

DISCUSSION

In the present study of patients with histologically diagnosed esophageal squamous cell carcinoma who underwent radical McKeown-type esophagectomy with at least two-field lymphadenectomy, we compared survival and complications in patients according to whether the RS or PM route was used. Our work had the advantages that it was performed in patients with the same disease etiology treated with a uniform therapeutic modality, and who underwent long-term follow-up focusing not only on postoperative complications but also on prognosis.

We confirmed that the reconstruction route was not associated with any significant variance in the extent of cardiac ($P = 0.494$) or pulmonary ($P = 0.214$) complications, as has been shown in previous studies[6,7,10].

The incidence of RLN palsy was 17.3% in our entire cohort, and did not differ between the PM and RS groups ($P = 0.707$). In esophagectomy patients, the

Table 4 Univariate and multivariate Cox's regression analysis predicting overall survival

Factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value[1]	HR (95% CI)	*P* value[1]
Age (yr)[2]	1.642 (1.097-2.460)	0.016	1.013 (0.991-1.035)	0.245
Sex[3]	0.690 (0.431-1.107)	0.124	0.690 (0.428-1.112)	0.127
BMI (kg/m^2)	0.974 (0.917-1.035)	0.400		
Smoking index[4]	1.499 (1.019-2.204)	0.040	1.000 (1.000-1.001)	0.596
Preoperative albumin level (g/L)	0.952 (0.917-0.988)	0.009	0.949 (0.912-0.987)	0.009
Tumor location[5]	0.872 (0.663-1.148)	0.330		
pT[6]	1.768 (1.209-2.585)	0.003	1.708 (1.163-2.508)	0.006
pN[7]	1.903 (1.571-2.306)	< 0.001	1.848 (1.525-2.239)	< 0.001
Tumor grade[8]	0.984 (0.731-1.324)	0.915		
Surgical bleeding	1.001 (0.999-1.002)	0.285		
Lymphadenectomy	0.655 (0.384-1.115)	0.119	0.779 (0.454-1.339)	0.367
Reconstruction[9]	1.157 (0.775-1.727)	0.477		

[1]Cox's proportional hazards model; [2]≤ 65 years of age *vs* > 65 years of age; [3]Male *vs* female; [4]≤ 400 *vs* > 400; [5]Upper thoracic *vs* middle thoracic *vs* lower thoracic; [6]pT1 *vs* pT2 *vs* pT3; [7]pN0 *vs* pN1 *vs* pN2 *vs* pN3; [8]G1 *vs* G2 *vs* G3; [9]Posterior mediastinal reconstruction *vs* retrosternal reconstruction. HR: Hazard ratio; pN: Pathological node; pT: Pathological tumor.

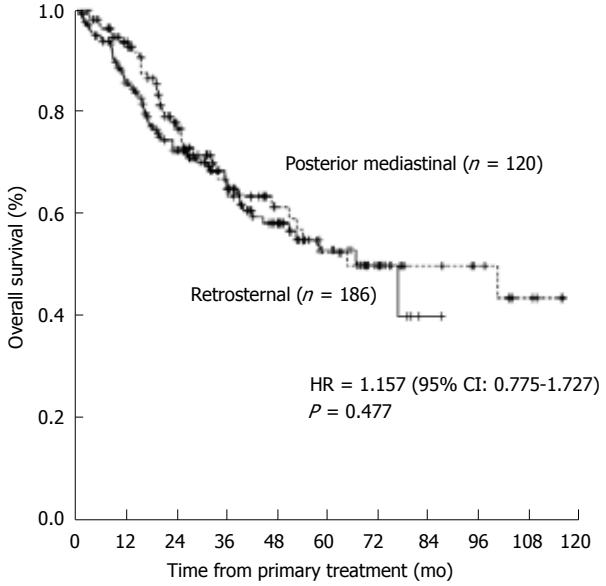


Figure 1 Kaplan-Meier curve showing overall patient survival stratified by route of reconstruction. HR: Hazard ratio.

prime etiology of RLN palsy is direct mechanical injury inflicted on the RLN during dissection[5,13]. Fang *et al*[14] reported that development of RLN palsy was closely linked to cervical dissection (22.9% *vs* 9.6%, $P = 0.089$) and anastomotic leakage (53.8% *vs* 13.5%, $P = 0.001$). These data lend support to our conclusion that surgical trauma and fistula-induced secondary corrosion play important roles in the development of RLN palsy (Table 3). Thus, RLN injury would be reduced if trauma during cervical lymphadenectomy were minimized and new anastomotic fistulae were managed in a timely manner.

The conduit is longer when the posterior route of reconstruction is used, therefore, a higher frequency of anastomotic leakage would be expected because a segment of stomach that is more remote from the blood supply is used (compared to the RS route)[8,9]. However, a recent meta-analysis by Urschel *et al*[10] showed that the reconstruction route chosen did not affect the frequency of anastomotic fistula (95% CI: 0.35-2.94; $P = 0.98$), in agreement with our data. Additionally, we found a significant association between anastomotic stricture and use of the RS route ($P = 0.018$). After review of the literature, we suggest that patients undergoing RS reconstruction are more at risk of anatomic stricture because of the narrow entrance to the thoracic inlet and the severe foregut angulation that are created when the RS route is used[7,11,15]. In this context, some authors recommend removal of the manubrium and the sternoclavicular joints[16,17]. We did not take these options; rather we prioritized thoracic stability and better patient appearance. Also, application of cervical anastomosis, which permits better intraoperative exposure at the cost of more severe postoperative pressure, has been reported to decrease fistula but increases stricture development[18]. This was not observed in our present work (anastomotic fistula, $P = 0.182$; anastomotic stricture, $P = 0.110$) (data not shown).

Chylothorax, the overall incidence of which was 6.2%, was significantly associated with use of the RS route ($P = 0.008$). However, prophylactic thoracic duct ligation or resection, which is known to mitigate against chylothorax[19,20], was more likely to be performed in patients of the PM group ($P < 0.001$). Additionally, each reconstructive option was determined by the preference of the individual surgeon. Therefore, surgeon characteristics and decisions regarding whether to perform thoracic duct ligation or resection seemed to be the major factors contributing to the higher incidence of chylothorax observed in the RS group. The route of reconstruction may not be important, in agreement with the data of previous studies[11,12].

Turning to surgical bleeding, Turnbull *et al*[21] considered that such bleeding was reduced when the PM technique was used because the extent of tunneling associated with this approach is less than that required when RS reconstruction is used. This is in line with the findings of the present study ($P < 0.001$). Although bleeding

often requires blood transfusion, the clinical significance of a 64.5-mL loss of blood is marginal.

In the present study, the 5-year OS rate was 50% (median: 26.8 mo), which was better than in previous studies[1,3]. This can be attributed to the strict enrolment criteria. Specifically, to facilitate objective comparisons, we excluded patients with pT4-stage disease, those with preoperative metastasis, and those who underwent non-radical (R1/R2) resections.

To date, the most commonly cited argument as to why the RS route of reconstruction should be favored is that it affords a radiotherapeutic advantage if local recurrence occurs[6,7], thus contributing to a favorable prognosis. However, to the best of our knowledge, no prior study has evaluated long-term survival in patients treated *via* the RS and PM reconstructive routes. Only the biological behavior of a tumor and the chosen treatment modality could influence survival. Thus, the objective of the present study was not to verify the independently prognostic significance of a given reconstructive route but to ascertain whether the route of reconstruction influenced the efficacy of a uniform treatment modality, thereby inducing a difference in survival. To explore this question, we performed prognostic analysis but failed to demonstrate any significant survival difference according to whether the RS or PM route was used (median OS, 25.4 mo *vs* 27.4 mo; $P = 0.477$) (Figure 1). Several possible explanations may be advanced. First, improvements in patient selection and surgical techniques, especially in the context of McKeown-type esophagectomy combined with extended lymphadenectomy, have decreased the rates of local recurrence[4,7,22]. Second, various treatment modalities (chemotherapy and/or radiotherapy and/or surgical resection) have been shown to be satisfactory in terms of efficacy and to allow acceptable levels of post-recurrence survival[23-26]. Thus, recurrence is no longer an intractable problem for which no effective treatment is available.

Several prognostic factors were identified upon uni/multivariate survival analysis; these included preoperative albumin level and pathological stage (pT and pN status). Lower preoperative albumin levels have long been regarded as indicative of poor nutritional status, abnormal liver function, and a metabolic response to acute phase disease[27,28]. A lower albumin level was significantly associated with a shorter survival time ($P = 0.009$). A similar result was reported by Lien *et al*[29] in patients with adenocarcinoma of the gastric cardia. The importance of pathological stage, the best-established prognostic factor for malignant disease, was emphasized once again in our present work.

The present study has both strengths and weaknesses. The work was retrospective in nature. All work was performed in a single institution, with patients who had disease of uniform etiology and who were identically treated. However, the data may be biased to some extent, because we could not control for surgical experience or guarantee that all documentation was completely accurate. However, we attempted to minimize the latter possible source of error by using consistent definitions when performing our review of records, and all data were independently checked.

In conclusion, this is believed to be the first retrospective study to investigate systematically the influence of reconstructive route on both clinical complications and prognosis. Use of the PM route of reconstruction reduces the incidence of postoperative complications, without compromising survival, and must be recommended for use in patients with resectable ESCC.

COMMENTS

Background

For patients with localized esophageal cancer, subtotal esophagectomy with a thoracic-abdominal-cervical incision (McKeown-type esophagectomy), combined with extensive lymphadenectomy, is now generally recognized as the optimal treatment in terms of long-term survival. Reconstruction under such circumstances commonly uses either the posterior mediastinal (PM) or retrosternal (RS) route. Despite studied and debated for decades, there remains no consensus as to the optimal route of reconstruction after subtotal esophagectomy.

Research frontiers

Postoperative complications and quality of life are hot topics and have been analyzed in previous studies. A recent meta-analysis showed that PM and anterior mediastinal routes of reconstruction are associated with similar outcomes.

Innovations and breakthroughs

To date, the most commonly cited argument as to why the RS route of reconstruction should be favored is that it affords a radiotherapeutic advantage if local recurrence occurs, thus contributing to a favorable prognosis. However, no prior study has evaluated long-term survival and, their results may be biased based on their small sample size. The authors' work had the advantages that it was performed in a large cohort with the same disease etiology treated with a uniform therapeutic modality, and who underwent long-term follow-up focusing not only on postoperative complications but also on prognosis.

Applications

Use of the PM route of reconstruction reduces the incidence of postoperative complications but does not affect survival. This route is recommended for treatment of patients with resectable esophageal squamous cell carcinoma.

Peer review

This paper is well written. The authors retrospectively reviewed 306 consecutive patients. They described that choice of reconstruction method was determined by surgeon preference, because there are still no criteria. Therefore, the surgeon factor should be included in all analyses. Especially in the analysis of risk for postesophagectomy chylothorax, the major factor seemed to be the surgeon and not route of reconstruction.

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BRIEF ARTICLE

Carbon dioxide insufflation for endoscopic retrograde cholangiopancreatography: A meta-analysis and systematic review

Yao Cheng, Xian-Ze Xiong, Si-Jia Wu, Jiong Lu, Yi-Xin Lin, Nan-Sheng Cheng, Tai-Xiang Wu

Yao Cheng, Xian-Ze Xiong, Si-Jia Wu, Jiong Lu, Yi-Xin Lin, Nan-Sheng Cheng, Department of Bile Duct Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Tai-Xiang Wu, Chinese Cochrane Centre, Chinese Clinical Trial Registry, Chinese Evidence-Based Medicine Centre, INCLEN Resource and Training Centre, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Author contributions: Cheng Y and Cheng NS designed the study; Wu SJ and Lu J performed the literature search and collected the data; Lin YX and Wu TX analyzed the data; Cheng Y and Xiong XZ wrote the paper.
Correspondence to: Nan-Sheng Cheng, MD, Professor, Department of Bile Duct Surgery, West China Hospital, Sichuan University, No. 37 Guo Xue Xiang, Chengdu 610041, Sichuan Province, China. nanshengcheng@gmail.com
Telephone: +86-28-85422465 Fax: +86-28-85422465
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Abstract

AIM: To assess the safety and efficacy of carbon dioxide (CO_2) insufflation during endoscopic retrograde cholangiopancreatography (ERCP).

METHODS: The Cochrane Library, Medical Literature Analysis and Retrieval System Online, Excerpta Medica Database, Science Citation Index Expanded, Chinese Biomedical Literature Database, and references in relevant publications were searched up to December 2011 to identify randomized controlled trials (RCTs) comparing CO_2 insufflation with air insufflation during ERCP. The trials were included in the review irrespective of sample size, publication status, or language. Study selection and data extraction were performed by two independent authors. The meta-analysis was performed using Review Manager 5.1.6. A random-effects model was used to analyze various outcomes. Sensitivity and subgroup analyses were performed if necessary.

RESULTS: Seven double-blind RCTs involving a total of 818 patients were identified that compared CO_2 insufflation (n = 404) with air insufflation (n = 401) during ERCP. There were a total of 13 post-randomization dropouts in four RCTs. Six RCTs had a high risk of bias and one had a low risk of bias. None of the RCTs reported any severe gas-related adverse events in either group. A meta-analysis of 5 RCTs (n = 459) indicated that patients in the CO_2 insufflation group had less post-ERCP abdominal pain and distension for at least 1 h compared with patients in the air insufflation group. There were no significant differences in mild cardiopulmonary complications [risk ratio (RR) = 0.43, 95% CI: 0.07-2.66, P = 0.36], cardiopulmonary (e.g., blood CO_2 level) changes [standardized mean difference (SMD) = -0.97, 95% CI: -2.58-0.63, P = 0.23], cost analysis (mean difference = 3.14, 95% CI: -14.57-20.85, P = 0.73), and total procedure time (SMD = -0.05, 95% CI: -0.26-0.17, P = 0.67) between the two groups.

CONCLUSION: CO_2 insufflation during ERCP appears to be safe and reduces post-ERCP abdominal pain and discomfort.

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Key words: Systematic review; Meta-analysis; Carbon dioxide insufflation; Endoscopic retrograde cholangiopancreatography; Abdominal pain

Peer reviewers: Yoshiharu Motoo, Professor, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan; Dr. Francesco Manguso, Gastroenterology Unit, AORN A Cardarelli, Via A. Cardarelli 9, 80122 Napoli, Italy

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) refers to radiographic visualization of the pancreatobiliary system by retrograde injection of contrast media through the ampulla of Vater[1]. ERCP, which was first introduced in the late 1960s[2], is now widely performed by endoscopists to diagnose various pancreatic and biliary diseases[1,3]. Although the exact number of patients undergoing ERCP worldwide each year is unknown, it has been reported that approximately 54 000 and 500 000 patients undergo ERCP annually in the United Kingdom and United States, respectively[4-6]. ERCP has become an invaluable tool in the diagnosis of numerous pancreatic and biliary diseases and is considered to be the gold standard study for the pancreatobiliary system[1,3-5].

Gas is deliberately insufflated into the bowel lumen during ERCP to provide operating space to ensure adequate visualization by the camera and manipulation of instruments in the duodenum[1-4]. Currently, air is the most commonly used gas for insufflation during ERCP worldwide[6]. However, air has some potential disadvantages. Air is not absorbed by the bowel and must be passed from the gastrointestinal tract in the form of flatus, which may lead to post-ERCP abdominal pain and discomfort (e.g., abdominal distension) because of gas retention in the gastrointestinal tract[7]. Recently, carbon dioxide (CO_2) has been introduced as an alternative to air for insufflation during ERCP[8-14].

CO_2 is rapidly absorbed from the bowel and is delivered directly to the lungs by the circulation[15,16]. Ultimately, it is excreted by the lungs during respiratory exchange[15,16]. Theoretically, CO_2 has the potential to reduce post-ERCP abdominal pain and discomfort due to lower gas retention[15,16]. However, the absorption of CO_2 may cause hypercapnia and acidosis, which must be prevented through hyperventilation[17]. The absorption of CO_2 may be associated with various cardiopulmonary side effects such as tachycardia, cardiac arrhythmias, hypoxemia, and pulmonary edema[17]. Elderly patients with cardiopulmonary diseases are more likely to suffer from these adverse events[9,17].

The use of CO_2 insufflation during ERCP is controversial. Some authors suggest that the application of CO_2 insufflation reduces post-ERCP abdominal pain and discomfort[8,11,12,14], thus they recommend the use of CO_2 in ERCP, whereas others do not think so[9,10,13]. To date, we have been unable to identify any meta-analysis that assesses the role of carbon dioxide insufflation during ERCP. We conducted a meta-analysis and systematic review to assess the safety and efficacy of CO_2 insufflation during ERCP for reduction of post-ERCP abdominal pain and discomfort.

MATERIALS AND METHODS

Identification of trials and data extraction

We searched the following databases up to December 2011 to identify randomized controlled trials (RCTs): The Cochrane Library, Medical Literature Analysis and Retrieval System Online (MEDLINE), Excerpta Medica Database (EMBASE), Science Citation Index Expanded, and Chinese Biomedical Literature Database (CBM). Search strategies for these databases are shown in Table 1. We also hand searched the references in relevant publications to explore additional relevant clinical trials. After completing all searches, we merged the search results using the software package Endnote X4 (reference management software) and removed duplicated records. Two independent authors (Lin YX and Xiong XZ) scanned the title and abstract of every record identified by the searches for inclusion. If compliance with inclusion criteria was not clear from the abstract, we retrieved full texts for further assessment. Only RCTs comparing CO_2 insufflation with air insufflation during ERCP were considered for the review, irrespective of sample size, publication status, or language. Two independent authors (Wu SJ and Lu J) independently extracted and confirmed the data and entered them into an electronic data collection form. We resolved all differences between authors by discussion.

Outcomes

Data for the following outcomes were extracted: abdominal pain (pain scores *via* visual analogue scale and number of pain-free patients at various time points after ERCP), abdominal distention, gas-related complications (severe gas-related adverse events and mild cardiopulmonary complications), cardiopulmonary changes (heart rate, blood pressure, blood pH, *etc.*), cost analysis, and total procedure time.

Assessment of methodological quality

Two authors (Cheng Y and Cheng NS) independently assessed the methodological quality of the included trials using the quality checklist recommended by the Cochrane Handbook[18]. We assessed the risk of bias of the trials based on the following domains: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting and other sources of bias[18]. Following the evaluation of the above domains, an included trial was judged as a trial with a low risk of bias if it was evaluated as "low" in all of the above domains. If the risk of bias was judged as "unclear" or "high", then the trial was listed under the group of trials with "high risk of bias". We resolved all disagreements by discussion and referral to a third author (Wu TX) for adjudication.

Statistical analysis

We performed the meta-analysis using the software pack-

Table 1 Search strategies

Databases	Period of search	Search strategies
The Cochrane library	Until 1st December 2011	1. MeSH descriptor Cholangiopancreatography, Endoscopic Retrograde explode all trees 2. (endoscopic retrograde cholangiopancreatograph*): ti, ab, kw OR (ERCP): ti, ab, kw 3. MeSH descriptor Carbon Dioxide explode all trees 4. (carbon dioxide): ti, ab, kw OR (CO_2): ti, ab, kw 5. 1 OR 2 6. 3 OR 4 7. 5 AND 6
MEDLINE *via* PubMed	Until 1st December 2011	1. "Cholangiopancreatography, Endoscopic Retrograde" [MeSH] OR endoscopic retrograde cholangiopancreatograph* [tiab] OR ERCP [tiab] 2. "Carbon Dioxide" [Mesh] OR carbon dioxide [tiab] OR CO_2 [tiab] 3. 1 AND 2
EMBASE *via* embase.com	Until 1st December 2011	1. 'endoscopic retrograde cholangiopancreatography'/exp OR 'endoscopic retrograde cholangiopancreatography' 2. 'ercp'/exp OR ercp 3. 'carbon dioxide'/exp OR 'carbon dioxide' 4. 'CO_2'/exp OR CO_2 5. 1 OR 2 6. 3 OR 4 7. 5 AND 6
Science citation index expanded	Until 1st December 2011	1. TS = ('endoscopic retrograde cholangiopancreatograph*' OR ERCP) 2. TS = ('carbon dioxide' OR CO_2) 3. 1 AND 2
CBM	Until 1st December 2011	Search strategy in Chinese. Includes search terms similar to the terms used in MEDLINE

MEDLINE: Medical Literature Analysis and Retrieval System Online; EMBASE: Excerpta Medica Database; CBM: Chinese Biomedical Literature Database; MeSH: Medical Subject Heading; ERCP: Endoscopic retrograde cholangiopancreatography.

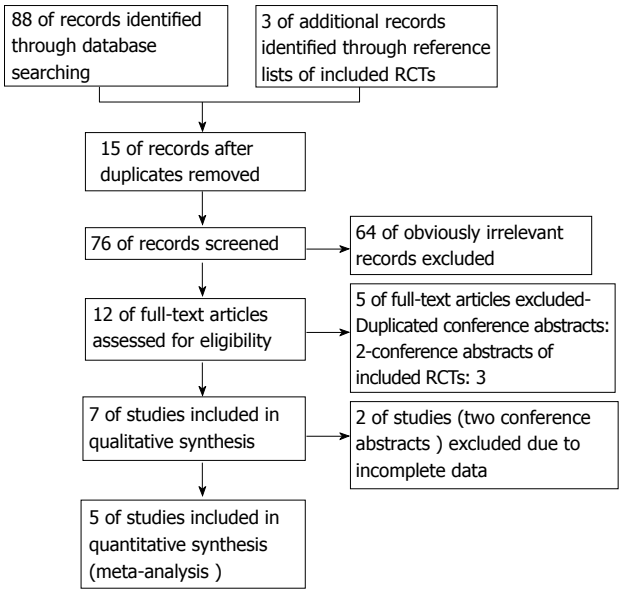


Figure 1 Flow diagram showing study selection process. RCT: Randomized controlled trial.

age Review Manager 5.1.6. Two authors (Wu SJ, Lu J) confirmed and entered all data into Review Manager independently. For dichotomous outcomes, we calculated the risk ratio (RR) with 95% CI[19]. For continuous outcomes, we calculated the mean difference (MD) with 95% CI[19]. For continuous outcomes with different measurement scales in different RCTs, we calculated the standardized mean difference (SMD) with 95% CI[19]. We described the heterogeneity with the Chi-squared test[19]. A *P* value less than 0.10 was considered to be significant heterogeneity[19]. We also used the I^2 statistic to measure the quantity of heterogeneity[19]. For all analysis, we employed the random-effects model. We intended to perform funnel plots and assessed their visual asymmetry to determine reporting biases[20]. In case of missing data, we contacted the original investigators to request further information. If there was no reply, we performed the analysis on an "intention-to-treat" (ITT) principle, if applicable[21]. Otherwise, we adopted the available-case analysis (also known as per-protocol analysis and PP analysis). We conducted the meta-analysis and systematic review according to the Cochrane Handbook for Systematic Reviews of Interventions[22] and Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA)[23].

RESULTS

Search results

We identified a total of 88 records through electronic searches of The Cochrane Library (n = 4), MEDLINE (n = 16), EMBASE (n = 47), Science Citation Index Expanded (n = 20), Chinese Biomedical Literature Database (n = 1), and hand search of the references of the included RCTs (n = 3). We excluded 15 duplicates and 64 clearly irrelevant records by reading titles and abstracts. Twelve full-text articles were retrieved for further assessment. We excluded five articles for the reasons listed in Figure 1.

Description of included trials

Seven RCTs (5 articles[8-12] and 2 conference abstracts[13,14]), which were published between 2007 and 2011, were identified that fulfilled the inclusion criteria. A total of

Table 2 Study characteristics

Author	Year	Country	Study design	Participants	Participants (CO_2/Air)	Mean age (CO_2/Air)
Bretthauer *et al*[8]	2007	Norway	Multi-centers	Low risk	118 (58/58)	57/54
Maple *et al*[12]	2009	United States	Single-center	Low risk	105 (50/50)	57/51.7
Dellon *et al*[9]	2010	United States	Single-center	High risk and low risk	78 (36/38)	60.1/59.7
Kuwatani *et al*[10]	2011	Japan	Multi-centers	Low risk	80 (40/40)	66.1/68.7
Luigiano *et al*[11]	2011	Italy	Single-center	Low risk	78 (39/37)	66.1/67.1
Mei *et al*[13]	2011	Australia	Single-center	Not mentioned	61 (34/27)	Not mentioned
Arjunan *et al*[14]	2011	India	Single-center	Low risk	298 (147/151)	Not mentioned

Low risk refers to patients without chronic obstructive pulmonary diseases; high risk refers to patients with chronic obstructive pulmonary diseases.

Table 3 Risk of bias in included trials

Studies	Sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting
Bretthauer *et al*[8]	Low risk	Low risk	Low risk	Low risk	High risk	High risk
Maple *et al*[12]	Low risk	Low risk	Low risk	Low risk	High risk	High risk
Dellon *et al*[9]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Kuwatani *et al*[10]	Low risk	Unclear risk	Low risk	Low risk	Low risk	High risk
Luigiano *et al*[11]	Low risk	Low risk	Low risk	Low risk	High risk	High risk
Mei *et al*[13]	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	Unclear risk
Arjunan *et al*[14]	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	Unclear risk

818 patients were included: 404 were assigned to the CO_2 group and 401 were allocated to the air group (13 post-randomization dropouts). Details on the included studies are shown in Table 2.

Risk of bias of included studies

The risk of bias is summarized in Table 3. Six RCTs[8,10-14] had a high risk of bias and one RCT[9] had a low risk of bias. All trials were double-blind RCTs with a parallel group study design. There were a total of 13 post-randomization dropouts in four RCTs[8,9,11,12] which were not included in the analysis. Only one RCT[9] reported costs.

Effects of interventions

Abdominal pain scores (intensity of pain): Seven RCTs reported the abdominal pain scores. Pain was measured with a 10-point visual analogue scale (VAS)[10,12-14] or a 100-mm VAS[8-9,11] at various time points, including before the procedure, during the procedure, 30 min, 1 h, 90 min, 3 h, 6 h and 24 h after ERCP. Four RCTs[8,11,12,14] showed decreased post-ERCP abdominal pain scores in the CO_2 group compared with the air group, while the other three RCTs[9-10,13] did not. The meta-analysis of 5 RCTs[8-12] showed that the abdominal pain scores 1 h after ERCP was significantly lower in the CO_2 group than the air group (I^2 = 98%; SMD = -1.81, 95% CI: -3.58--0.04, P = 0.04). The pain scores 3 h, 6 h and 24 h after ERCP were also lower in the CO_2 group than in the air group, but the differences were not significant (Figure 2A).

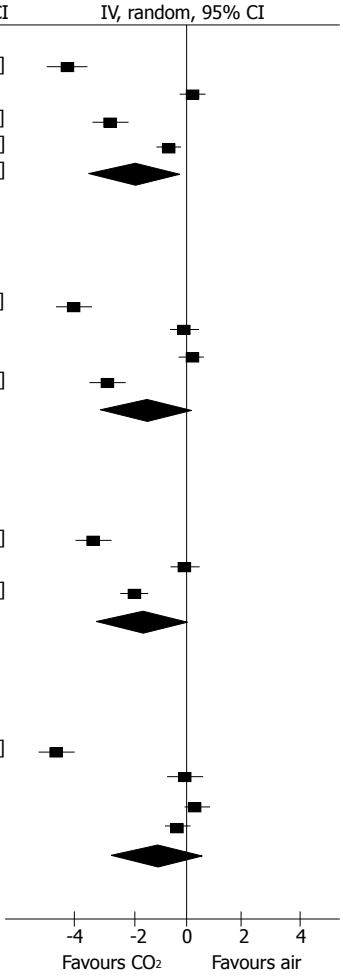
Pain-free patients (incidence of pain): Only two RCTs[8,12] reported the number of pain-free patients at various time points, including 1, 3, 6 and 24 h after ERCP. The meta-analysis showed that the rate of pain-free patients 1 h and 6 h after ERCP was significantly higher in the CO_2 group than in the air group: (I^2 = 74%; RR = 1.79, 95% CI: 1.01-3.20, P = 0.05) and (RR = 2.22, 95% CI: 1.37-3.62, P = 0.001), respectively. The rate of pain-free patients 3 h and 24 h after ERCP were also higher in the CO_2 group than in the air group, but the differences were not significant (Figure 2B).

Abdominal distention: Abdominal distension was measured with a 100-mm VAS in two RCTs[10,11]. The meta-analysis showed that abdominal distension 1 h after ERCP was significantly lower in the CO_2 group than in the air group (SMD = -1.43, 95% CI: -1.93--0.92, P < 0.00001). There was no significant difference in abdominal distension between the two groups at 3 h, 6 h and 24 h after ERCP (Figure 2C). Three RCTs[9,12,14] reported the increase in abdominal girth. Maple *et al*[12] and Arjunan *et al*[14] stated that CO_2 insufflation was associated with less increase in abdominal girth than air insufflation. However, Dellon *et al*[9] indicated that there was no significant difference between groups with regard to increase in abdominal girth. One RCT[8] reported the number of patients with bowel distention as assessed by X-ray photographs. There were fewer patients with bowel distention in the CO_2 group than in the air group (RR = 0.76, 95% CI: 0.60-0.98, P = 0.03).

Complications: None of the RCTs reported any severe gas-related adverse events in either group (e.g., death, embolism, cardiac arrhythmias, and significant respiratory events). Mild cardiopulmonary complications (e.g., respiratory depression, hypotension, and bradycardia) were reported in five RCTs[8-12]. There was no significant difference in the number of patients with any mild car-

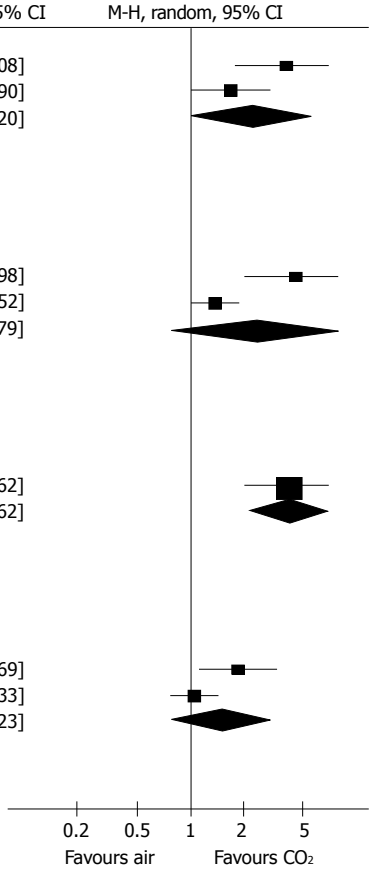
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Study or subgroup	CO_2 Mean	CO_2 SD	CO_2 Total	Air Mean	Air SD	Air Total	Weight	SMD IV, random, 95% CI
1.1.1 Pain scores *via* VAS (1 h after ERCP)								
Bretthauer 2007	5.7	1.9	39	19.2	3.8	52	24.5%	-4.27 [-5.03, -3.51]
Dellon 2010	16.4	25.2	36	10.8	19.3	38	25.3%	0.25 [-0.21, 0.71]
Luigiano 2011	10.0	4.4	37	35.0	12.0	39	24.9%	-2.71 [-3.34, -2.08]
Maple 2009	0.7	2.0	50	1.9	2.0	50	25.4%	-0.60 [-1.00, -0.19]
Subtotal (95% CI)			162			179	100.0%	-1.81 [-3.58, -0.04]
Heterogeneity: Tau^2 = 3.16; χ^2 = 131.64, df = 3 (P < 0.00001); I^2 = 98%								
Test for overall effect: Z = 2.01 (P = 0.04)								
1.1.2 Pain scores *via* VAS (3 h after ERCP)								
Bretthauer 2007	7.6	2.4	39	21.2	3.9	52	24.7%	-4.03 [-4.76, -3.30]
Dellon 2010	20.8	32.2	32	22.3	27.8	34	25.2%	-0.05 [-0.53, 0.43]
Kuwatani 2011	1.4	2.0	40	0.9	2.0	40	25.3%	0.25 [-0.19, 0.69]
Luigiano 2011	8.0	2.5	37	28.1	9.6	39	24.9%	-2.80 [-3.45, -2.16]
Subtotal (95% CI)			148			165	100.0%	-1.64 [-3.55, 0.27]
Heterogeneity: Tau^2 = 3.71; χ^2 = 142.60, df = 3 (P < 0.00001); I^2 = 98%								
Test for overall effect: Z = 1.69 (P = 0.09)								
1.1.3 Pain scores *via* VAS (6 h after ERCP)								
Bretthauer 2007	9.8	3.1	39	22.1	4.1	52	33.0%	-3.29 [-3.93, -2.65]
Dellon 2010	18.3	25.4	32	19.5	26.7	34	33.6%	-0.05 [-0.53, 0.44]
Luigiano 2011	7.0	2.5	37	14.1	4.7	39	33.4%	-1.85 [-2.40, -1.31]
Subtotal (95% CI)			108			125	100.0%	-1.72 [-3.55, 0.11]
Heterogeneity: Tau^2 = 2.54; χ^2 = 66.27, df = 2 (P < 0.00001); I^2 = 97%								
Test for overall effect: Z = 1.84 (P = 0.07)								
1.1.4 Pain scores *via* VAS (24 h after ERCP)								
Bretthauer 2007	3.9	1.0	39	19.8	4.4	52	24.3%	-4.64 [-5.45, -3.84]
Dellon 2010	15.0	24.7	32	15.5	24.7	34	25.2%	-0.02 [-0.50, 0.46]
Kuwatani 2011	1.1	1.9	40	0.5	0.9	40	25.3%	0.40 [-0.04, 0.84]
Luigiano 2011	4.2	3.4	37	5.0	2.8	37	25.3%	-0.25 [-0.71, 0.20]
Subtotal (95% CI)			148			163	100.0%	-1.10 [-2.74, 0.55]
Heterogeneity: Tau^2 = 2.75; χ^2 = 122.05, df = 3 (P < 0.00001); I^2 = 98%								
Test for overall effect: Z = 1.30 (P = 0.19)								
Test for subgroup differences: χ^2 = 0.42, df = 3 (P = 0.94), I^2 = 0%								



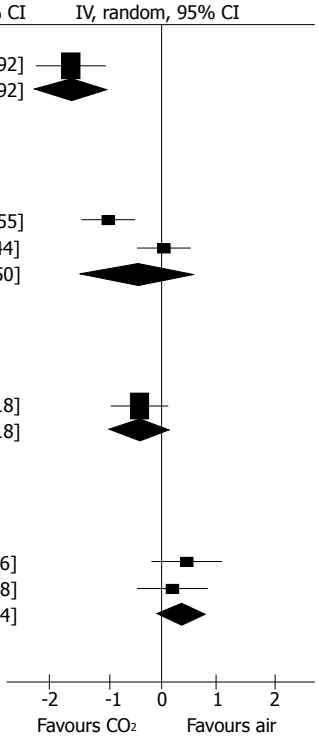
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Study or subgroup	CO_2 Events	CO_2 Total	Air Events	Air Total	Weight	Risk ratio M-H, random, 95% CI
1.2.1 Pain-free patients (1 h after ERCP)						
Bretthauer 2007	26	39	14	52	44.5%	2.48 [1.50, 4.08]
Maple 2009	36	50	26	50	55.5%	1.38 [1.01, 1.90]
Subtotal (95% CI)		89		102	100.0%	1.79 [1.01, 3.20]
Total events	62		40			
Heterogeneity: Tau^2 = 0.13; χ^2 = 3.87, df = 1 (P = 0.05); I^2 = 74%						
Test for overall effect: Z = 1.98 (P = 0.05)						
1.2.2 Pain-free patients (3 h after ERCP)						
Bretthauer 2007	26	39	12	52	46.5%	2.89 [1.68, 4.98]
Maple 2009	45	50	36	50	53.5%	1.25 [1.03, 1.52]
Subtotal (95% CI)		89		102	100.0%	1.85 [0.71, 4.79]
Total events	71		48			
Heterogeneity: Tau^2 = 0.43; χ^2 = 10.94, df = 1 (P = 0.0009); I^2 = 91%						
Test for overall effect: Z = 1.26 (P = 0.21)						
1.2.3 Pain-free patients (6 h after ERCP)						
Bretthauer 2007	25	39	15	52	100.0%	2.22 [1.37, 3.62]
Subtotal (95% CI)		39		52	100.0%	2.22 [1.37, 3.62]
Total events	25		15			
Heterogeneity: Not applicable						
Test for overall effect: Z = 3.21 (P = 0.001)						
1.2.4 Pain-free patients (24 h after ERCP)						
Bretthauer 2007	25	39	19	52	44.1%	1.75 [1.14, 2.69]
Maple 2009	38	50	36	50	55.9%	1.06 [0.84, 1.33]
Subtotal (95% CI)		89		102	100.0%	1.32 [0.78, 2.23]
Total events	63		55			
Heterogeneity: Tau^2 = 0.11; χ^2 = 4.65, df = 1 (P = 0.03); I^2 = 78%						
Test for overall effect: Z = 1.05 (P = 0.30)						
Test for subgroup differences: χ^2 = 2.06, df = 3 (P = 0.56), I^2 = 0%						



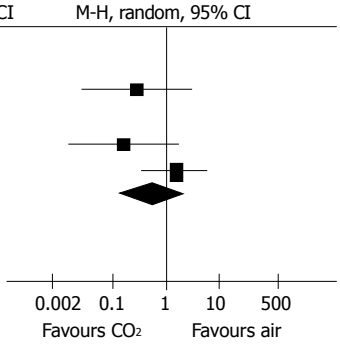
C

Study or subgroup	CO_2 Mean	CO_2 SD	CO_2 Total	Air Mean	Air SD	Air Total	Weight	SMD IV, random, 95% CI
1.3.1 Abdominal distension *via* VAS (1 h after ERCP)								
Luigiano 2011	7.2	3.6	37	23.1	15.0	39	100.0%	-1.43 [-1.93, -0.92]
Subtotal (95% CI)			37			39	100.0%	-1.43 [-1.93, -0.92]
Heterogeneity: Not applicable								
Test for overall effect: Z = 5.52 (P < 0.00001)								
1.3.2 Abdominal distension *via* VAS (3 h after ERCP)								
Luigiano 2011	6.1	2.1	37	13.1	9.2	39	49.5%	-1.03 [-1.51, -0.55]
Kuwatani 2011	0.6	1.2	40	0.6	1.1	40	50.5%	0.00 [-0.44, 0.44]
Subtotal (95% CI)			77			79	100.0%	-0.51 [-1.51, 0.50]
Heterogeneity: Tau^2 = 0.47; χ^2 = 9.57, df = 1 (P = 0.002); I^2 = 90%								
Test for overall effect: Z = 0.99 (P = 0.32)								
1.3.3 Abdominal distension *via* VAS (6 h after ERCP)								
Luigiano 2011	4.7	4.5	37	6.2	6.1	39	100.0%	-0.28 [-0.73, 0.18]
Subtotal (95% CI)			37			39	100.0%	-0.28 [-0.73, 0.18]
Heterogeneity: Not applicable								
Test for overall effect: Z = 1.20 (P = 0.23)								
1.3.4 Abdominal distension *via* VAS (24 h after ERCP)								
Luigiano 2011	3.1	2.5	37	2.2	3.2	39	48.5%	0.31 [-0.14, 0.76]
Kuwatani 2011	0.6	1.6	40	0.4	1.1	40	51.5%	0.14 [-0.29, 0.58]
Subtotal (95% CI)			77			79	100.0%	0.22 [-0.09, 0.54]
Heterogeneity: Tau^2 = 0.00; χ^2 = 0.26, df = 1 (P = 0.61); I^2 = 0%								
Test for overall effect: Z = 1.39 (P = 0.16)								
Test for subgroup differences: χ^2 = 29.66, df = 3 (P < 0.00001), I^2 = 89.9%								



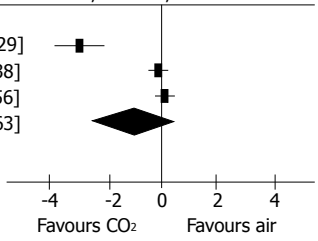
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Study or subgroup	CO_2 Events	CO_2 Total	Air Events	Air Total	Weight	Risk ratio M-H, random, 95% CI
1.4.1 Mild cardiopulmonary complications						
Bretthauer 2007	0	58	0	58		Not estimable
Dellon 2010	0	36	2	38	25.6%	0.21 [0.01, 4.25]
Kuwatani 2011	0	40	0	40		Not estimable
Luigiano 2011	0	37	5	39	27.3%	0.10 [0.01, 1.67]
Maple 2009	3	50	2	50	47.1%	1.50 [0.26, 8.60]
Subtotal (95% CI)		221		225	100.0%	0.43 [0.07, 2.66]
Total events	3		9			
Heterogeneity: Tau^2 = 1.05; χ^2 = 3.30, df = 2 (P = 0.19); I^2 = 39%						
Test for overall effect: Z = 0.91 (P = 0.36)						
Test for subgroup differences: Not applicable						



E

Study or subgroup	CO_2 Mean	CO_2 SD	CO_2 Total	Air Mean	Air SD	Air Total	Weight	SMD IV, random, 95% CI
1.5.1 Change in blood CO_2 level								
Bretthauer 2007	0.3	0.2	28	1.1	0.3	34	32.3%	-3.04 [-3.79, -2.29]
Dellon 2010	8.8	4.8	36	9.4	10.1	38	33.8%	-0.07 [-0.53, 0.38]
Luigiano 2011	2.4	1.9	37	2.2	1.8	39	33.8%	0.11 [-0.34, 0.56]
Subtotal (95% CI)			101			111	100.0%	-0.97 [-2.58, 0.63]
Heterogeneity: Tau^2 = 1.92; χ^2 = 54.85, df = 2 (P < 0.00001); I^2 = 96%								
Test for overall effect: Z = 1.19 (P = 0.23)								
Test for subgroup differences: Not applicable								



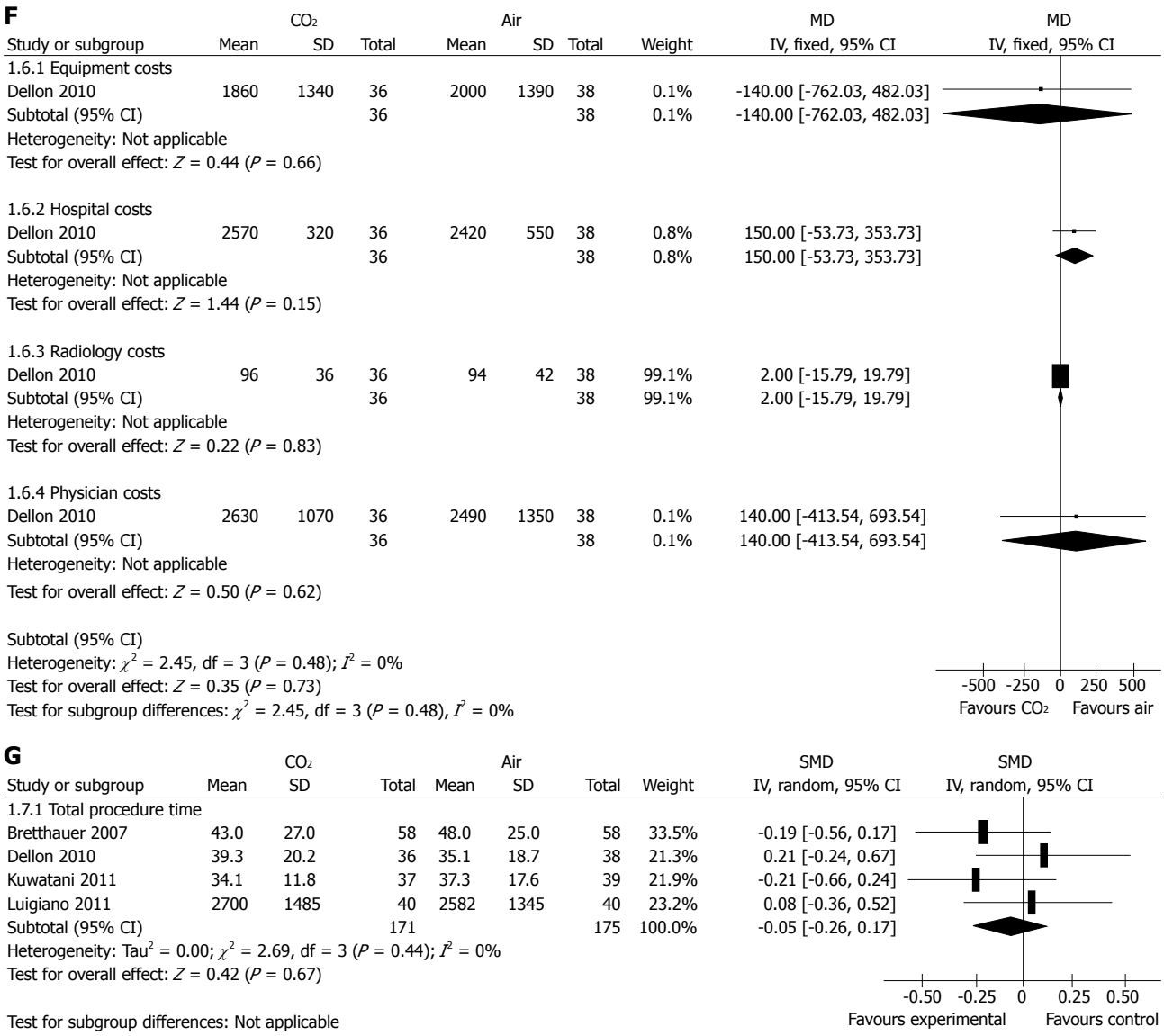


Figure 2 Forest plots of meta-analysis. A: Carbon dioxide *vs* air in abdominal pain scores; B: Carbon dioxide *vs* air in pain-free patients; C: Carbon dioxide *vs* air in abdominal distension; D: Carbon dioxide *vs* air in mild cardiopulmonary complications; E: Carbon dioxide *vs* air in change in blood carbon dioxide level; F: Carbon dioxide *vs* air in total costs; G: Carbon dioxide *vs* air in total procedure time. IV: Inverse-variance; M-H: Mantel Haenszel; ERCP: Endoscopic retrograde cholangiopancreatography; VAS: Visual analogue scale; MD: Mean difference; SMD: Standardized mean difference.

diopulmonary complications between groups (I^2 = 39%; RR = 0.43, 95% CI: 0.07-2.66, P = 0.36) (Figure 2D).

Cardiopulmonary changes: Changes in blood CO_2 levels were reported in three RCTs[8,9,11]. There was no significant difference in the change in blood CO_2 level between the two groups (I^2 = 96%; SMD = -0.97, 95% CI: -2.58-0.63, P = 0.23). Only one RCT[10] reported blood oxygen saturation (SpO_2); Kuwatani *et al*[10] stated that there was no significant difference between the two groups at any time point (Figure 2E).

Cost analysis: Only one RCT[9] reported the cost analysis. There were no significant differences in the total costs for ERCP (I^2 = 0%; MD = 3.14, 95% CI: -14.57-20.85, P = 0.73), including equipment costs, hospital costs, radiology costs, and physician costs (Figure 2F).

Total procedure time: Four RCTs were included in the meta-analysis[8-11]. There was no significant difference in the total procedure time between the two groups (I^2 = 0%; SMD = -0.05, 95% CI: -0.26-0.17, P = 0.67). Maple *et al*[12] stated that there was no difference in total procedure time between the two groups (Figure 2G).

DISCUSSION

This systematic review with meta-analysis of RCTs assessed the safety and efficacy of CO_2 insufflation *vs* air insufflation during ERCP for reduction of abdominal pain and discomfort. The meta-analysis of 5 RCTs indicated that CO_2 insufflation during ERCP was associated with less post-ERCP abdominal pain and distension for at least 1 h. There were no significant differences in mild cardiopulmonary complications, cardiopulmonary

changes, cost analysis, and total procedure time between the two groups.

Post-ERCP abdominal pain and discomfort are common in clinical practice[24]. Abdominal pain related to insufflation is nonspecific and may mimic the symptoms of severe post-ERCP complications, including pancreatitis and perforation, which can be a source of stress to both patients and endoscopists[9]. Some patients need hospitalization for further evaluation and observation of post-ERCP abdominal pain and discomfort[7,15]. Post-ERCP abdominal pain and discomfort may result from air insufflation, as air is difficult to dissolve in blood and stays in the bowel for several hours after ERCP[7,16]. CO_2 has unique characteristics in that it is cheap, colorless, nonflammable, non-explosive, easily excreted, and nontoxic to patients[16]. CO_2 insufflation during ERCP was first introduced by Bretthauer *et al*[8] in 2007. Bretthauer *et al*[8] found that CO_2 insufflation effectively reduced post-ERCP abdominal pain and discomfort and recommended its routine use in ERCP. Then the benefits of CO_2 insufflation were further demonstrated by three later RCTs[11,12,14]. On the contrary, three additional RCTs[9,10,13] showed no differences in post-ERCP abdominal pain or discomfort between CO_2 insufflation and air insufflation. Although this meta-analysis suggested that CO_2 insufflation was associated with less abdominal pain and discomfort for at least 1 h, it included only 5 RCTs and all had small sample sizes. Consequently, the data from three ongoing RCTs performed by Mei *et al*[13], Arjunan *et al*[14] and Janssens *et al*[15] (Trial number: UMIN000005755) are anticipated to resolve the controversy.

The safety of CO_2 insufflation during ERCP is another major concern for patients and endoscopists. Air insufflation is associated with rare but severe adverse events such as combustion when using electrocautery and gas embolisms[16,25]. CO_2 is non-flammable and more soluble than air. In theory, CO_2 insufflation is safer than air insufflation with regard to combustion and embolisms. CO_2 is the most commonly used gas for insufflation in laparoscopy. The safety of CO_2 insufflation for endoscopy has been well established in colonoscopy[16]. None of the included RCTs reported any severe CO_2-related adverse events (e.g., death, embolism, cardiac arrhythmias, or significant respiratory events). With regard to other adverse effects from CO_2 insufflation, the meta-analysis showed there were no differences in mild cardiopulmonary complications and cardiopulmonary changes between the two groups. However, all RCTs except one[9] excluded patients with chronic obstructive pulmonary diseases (COPD). The safety of CO_2 insufflation during ERCP for high-risk patients (e.g., patients with cardiopulmonary diseases or American Society of Anesthesiologists (ASA) Physical Status classification Ⅲ or Ⅳ) needs further evaluation.

There are other potential benefits of CO_2 insufflation for ERCP, such as the possibility of immediate computed tomography cholangiopancreatography after ERCP, which has recently been introduced to obtain clear images, and the possibility of intraoperative ERCP during laparoscopy[16,26].

The feasibility of CO_2 insufflation during ERCP is as follows. First, to date there have been three types of commercial CO_2 insufflators available: Olympus KeyMed ECR, Olympus UCR, and CO_2-EFFICIENT[15]. In addition, the cost of a CO_2 insufflator is not very high, at approximately 7000 euros[15]. CO_2 gas is inexpensive and convenient to obtain[15]. Dellon *et al*[9] found that the total costs for ERCP (including equipment costs, hospital costs, radiology costs, and physician costs), is similar between groups ($7170 in the CO_2 group *vs* $7000 in the air group). Moreover, the safety of CO_2 insufflation during ERCP has been well documented in the RCTs (see above). Thus, it appears that widespread implementation of CO_2 insufflation during ERCP is anticipated.

This review included a total of seven RCTs. Most excluded patients with COPD, whereas only one study[9] included patients with COPD. Thus, the results of this review may only be relevant to low-risk patients without cardiopulmonary diseases.

The overall quality of the current best evidence was low. Only one RCT had a low risk of bias. There were a total of 13 post-randomization dropouts in four RCTs. All four RCTs adopted the available-case analysis (also known as per-protocol analysis and PP analysis) without performing an intension-to-treat (ITT) analysis. Only one RCT reported the cost analysis. Further RCTs are anticipated to perform both PP analysis and ITT analysis in case of post-randomization dropouts (missing data) and report the costs of ERCP.

Dellon *et al*[7] conducted a systematic review published in 2009 which assessed the role of CO_2 insufflation for flexible sigmoidoscopy, colonoscopy, ERCP, and double-balloon enteroscopy. This review included nine RCTs, but only one used CO_2 insufflation for ERCP. In addition, the authors did not perform a meta-analysis because of obvious heterogeneity. Our findings are similar to the previous systematic review in terms of the safety of CO_2 insufflation. In contrast to our study, the previous systematic review showed a reduction in abdominal pain for at least 6 h in the CO_2 insufflation group when compared with the air group.

The limitations of our review are as follows. The first concerns the small number of included RCTs and the small sample size of each RCT. We included only seven RCTs with 404 patients undergoing ERCP with CO_2 insufflation; thus, there is a lack of available data on this issue to date. In addition, we did not perform funnel plots to assess the publication bias due to the small number of included RCTs.

This review currently provides the best available evidence for comparison of CO_2 insufflation versus air insufflation during ERCP. On the basis of this evidence, CO_2 insufflation during ERCP appears to be safe for the majority of patients and results in less post-ERCP abdominal pain and discomfort than air insufflation. Further RCTs with a low risk of bias and a greater number of patients are necessary to assess the role of CO_2

insufflation in high-risk patients. Future RCTs need to be conducted and reported according to the Consolidated Standards of Reporting Trials (CONSORT) statement[27].

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COMMENTS

Background

Endoscopic retrograde cholangiopancreatography (ERCP) is now widely performed by endoscopists to diagnose various pancreatic and biliary diseases. Currently, air is the most commonly used gas for insufflation during ERCP worldwide. However, air has some potential disadvantages.

Research frontiers

Recently, carbon dioxide (CO_2) has been introduced as an alternative to air for insufflation during ERCP. Many studies, including randomized controlled trials (RCTs), have been conducted to assess the safety and efficacy of CO_2 insufflation during ERCP for reduction of post-ERCP abdominal pain and discomfort. However, the use of CO_2 insufflation during ERCP is controversial.

Innovations and breakthroughs

The authors identified all RCTs comparing CO_2 insufflation with air insufflation during ERCP. They conducted a meta-analysis and systematic review according to the Cochrane Handbook for Systematic Reviews of Interventions and Preferred Reporting Items for Systematic reviews and Meta-Analysis. They found that CO_2 insufflation during ERCP appears to be safe and reduces post-ERCP abdominal pain and discomfort.

Applications

Due to reduced post-procedure abdominal pain and discomfort, CO_2 insufflation may be preferable to air insufflation during ERCP and should be recommended in clinical practice.

Terminology

ERCP refers to radiographic visualization of the bile duct and pancreatic duct by retrograde injection of contrast media into the pancreatobiliary system; RCTs refer to trials in which people are allocated at random to receive one of several clinical interventions.

Peer review

This is the first well-designed meta-analysis on the role of CO_2 insufflation in ERCP. The results are interesting and suggest that CO2 insufflation may be preferable to air insufflation during ERCP. However, this article has several limitations, such as a small number of RCTs.

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CASE REPORT

Clostridium perfringens bacteremia caused by choledocholithiasis in the absence of gallbladder stones

Antwan Atia, Tejas Raiyani, Pranav Patel, Robert Patton, Mark Young

Antwan Atia, Robert Patton, Mark Young, Departments of Gastroenterology, James H Quillen VA Medical Center, Johnson, Mountain Home, TN 37615, United States
Tejas Raiyani, Pranav Patel, General Internal Medicine, Quillen College of Medicine, East Tennessee State University, Johnson, Mountain Home, TN 37615, United States
Author contributions: Atia A managed patient while being hospitalized and he wrote the discussion section; Raiyani T and Patel P wrote the abstract, introduction and case part; Patton R managed the case and did revision of the manuscript; and Young M did final critical revision of the papers.
Correspondence to: Antwan Atia, MD, Departments of Gastroenterology, James H Quillen VA Medical Center, Johnson, Mountain Home, TN 37615,
United States. antwan.atia@yahoo.com
Telephone: +1-423-7477774 Fax: +1-423-7477774
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Abstract

A 67-years-old male presented with periumbilical abdominal pain, fever and jaundice. His anaerobic blood culture was positive for *clostridium perfringens*. Computed tomogram scan of the abdomen and abdominal ultrasound showed normal gallbladder and common bile duct (CBD). Subsequently magnetic resonance cholangiopancreaticogram showed choledocholithiasis. Endoscopic retrograde cholangiopancreaticogram-with sphincterotomy and CBD stone extraction was performed. The patient progressively improved with antibiotic therapy Choledocholithiasis should be considered as a source of *clostridium perfringens* bacteremia especially in the setting of elevated liver enzymes with cholestatic pattern.

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Key words: Choledocholithiasis; Clostridium perfringens; Bacteremia

Peer reviewer: Taketo Yamaguchi, MD, PhD, Vice Director, Department of Gastroenterology, Chiba Cancer Center, 666-2 Nitona-cho, Chuo-ku, Chiba 260-8717, Japan

Atia A, Raiyani T, Patel P, Patton R, Young M. Clostridium perfringens bacteremia caused by choledocholithiasis in the absence of gallbladder stones. *World J Gastroenterol* 2012; 18(39): 5632-5634 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i39/5632.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i39.5632

INTRODUCTION

Clostridium species are the second most common causes of anaerobic bacteremia, with *Clostridium perfringens* the most frequently isolated. Malignancies, gastrointestinal disorders and other chronic illnesses have been associated with *clostridium perfringens* bacteremia. *Clostridium perfringens* can cause food poisoning, gas gangrene, necrotizing enterocolitis, tuboovarian abscess, emphysematous cholecystitis, discitis, and liver abscess. Choledocholithiasis has rarely been reported as a source of *Clostridium perfringens* bacteremia. We describe a case of *clostridium perfringens* bacteremia caused by choledocholithiasis in the absence of gallbladder stones and with normal common bile duct (CBD) diameter and discuss management and review of the literature of this interesting but rare entity.

CASE REPORT

A 67-year-old male with medical history of coronary artery disease, diabetes mellitus, hypertension, hypothyroidism, chronic obstructive pulmonary disease (COPD) and diverticulosis presented with abdominal pain and fever. Fever with chills started four days prior to admission. Abdominal pain was sharp, periumbilical, non-radiating, lasted for 4 h, unrelated to food and resolved on its own. He denied nausea, vomiting, blood in stools but had chronic constipation. His last colonoscopy was

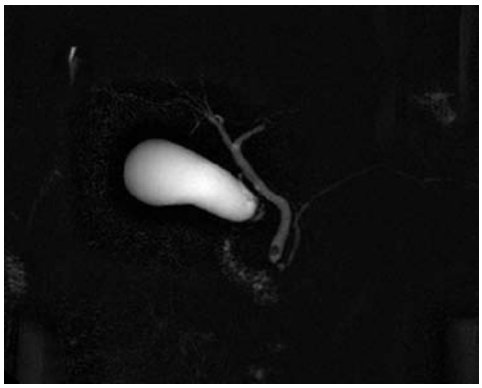


Figure 1 Magnetic resonance cholangiopancreatography shows filling defects within the distal common bile duct.

two years prior and showed sigmoid diverticulosis and colon polyps. On physical examination his heart rate was 103 and he had scleral icterus. The remainder of physical examination including abdominal examination was normal. Initial laboratory data showed white cell count of 8.2 (4.8-10.5), total bilirubin: 3 (0.2-1.0 mg/dL), direct bilirubin: 0.4 (0.0-0.2 mg/dL), aspartate transaminase: 131 (15-46 U/L), alanine transaminase: 86 (7-56 U/L) and alkaline phosphatase: 254 (38-126 U/L). His liver enzymes were normal one year earlier. Computed tomography (CT) scan of the abdomen and pelvis showed normal gallbladder, normal CBD diameter and colonic diverticulosis without diverticulitis. Ultrasound (US) of the abdomen revealed no stone in the gallbladder and the CBD diameter was 4.5 mm. Anaerobic blood culture was positive for clostridium perfringens. Patient was treated with vancomycin, aztreonam and metronidazole. He was allergic to penicillin. Urine culture was negative. Magnetic resonance cholangiopancreatography (MRCP) showed revealed a 6 mm ovoid filling defect and additional smaller filling defects within the distal common bile duct (Figure 1). Subsequently, endoscopic retrograde cholangiopancreatography confirmed the diagnosis of choledocholithiasis for which sphincterotomy and stone extraction was performed (Figure 2). Following the procedure, liver enzymes improved. He was discharged home on ertapenem and he had colonoscopy on outpatient basis that showed sigmoid diverticulosis.

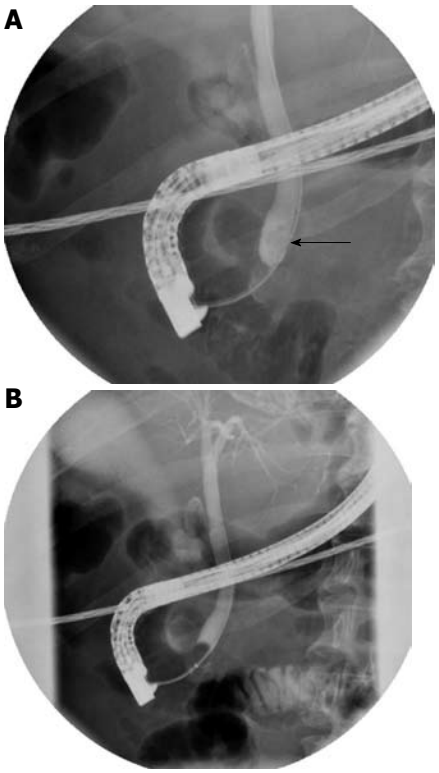


Figure 2 Endoscopic retrograde cholangiopancreatography. A: Filling defects within the distal common bile duct (arrow); B: Filling defects after sphincterotomy and ballon sweep.

DISCUSSION

Clostridium bacteremia is a rare occurrence that can lead to a devastating outcome. Early recognition and treatment of *clostridium* bacteremia can be life saving. The aim of this report is to present a rare case of *Clostridium perfringens* bacteremia caused by choledocholithiasis. There are few epidemiological studies documenting the incidence of *clostridium* bacteremia. A retrospective population-based surveillance for clostridial bacteremia among all residents of the Calgary Health Region (population 1.2 million) during 2000-2006 showed that the incidence of clostridium bacteremia was 1.8/100 000 per year. *Clostridium perfringens* was the most common isolate accounting for 42% of cases followed by *Clostridium Septicum, Clostridium ramosum, Clostridium clostridiiforme,* and *Clostridium difficile*[1]. Another review of blood cultures drawn in a rural hospital in Wisconsin, United States from 1990-1997 yielded clostridium infection in 0.12% of drawn cultures with *Clostridium perfringens* being the most common (21.7%)[2]. A review of blood cultures in a Japanese tertiary center during 2001-2009 showed that only 18 patients had *clostridium perfringens* bacteremia[3]. *Clostridium perfringens* is an anaerobic gram positive rod that can produce a wide spectrum of diseases related to toxin production. *Clostridium perfringens* produce four principal toxins including alpha, beta, epsilon, and iota toxins[4]. Alpha toxin can produce gas gangrene[5] while beta toxin can produce necrotic enteritis[6]. *Clostridium perfringens* has also been reported to cause tuboovarian abscess[7], necrotizing enterocolitis[8], emphysematous cholecystitis[9], discitis[10], and liver abscess[11]. *Clostridium perfringens* bacteremia has been reported to occur following colonoscopy and gynecological procedures[12,13]. Advanced age and co-morbidities such as hemodialysis, cancer, heart disease, diabetes, Crohn's disease, COPD, stroke and asthma increase the risk for clostridial infections. Advanced age increases the risk independent of co-morbidities which could be explained by age-related increase of clostridial species in the normal intestinal flora[1]. *Clostridium perfringens* bacteremia has been associated with intravascular hemolysis and death. Alpha toxin

produced by *clostridium perfringens* can cause hemolysis, platelet destruction and widespread capillary damage. Intravascular hemolysis can be fatal unless treatment is instituted early. Sudden severe hemolytic anaemia, very low MCV, hemolyzed blood samples and negative Coombs test in a patient with fever should prompt the clinician to consider *clostridium perfringens* infection. The morphological findings seen on blood cell examination are spherocytes, microspherocytes, and neutrophils with vacuoles or Dohle bodies[14-16]. Fortunately, our patient did not manifest these abnormalities. In our patient it is difficult to determine if the predominance of indirect hyperbilirubinemia is caused by intravascular hemolysis that was limited by early antibiotics use or by choledocholithiasis. Therefore we would encourage clinicians to obtain peripheral blood smear in patients with *clostridium perfringens* bacteremia with any evidence suggestive of intravascular hemolysis.

When the clinician encounters *clostridium perfringens* bacteremia, discovery of the source is very important. Sources of *Clostridium perfringens* bacteremia include colon, biliary tree, lungs, tuboovarian, endometrium and decubitus ulcer. Unlikely sources include urinary tract, pancreas, small bowel, esophagus and brain abscess or unknown source[2]. Choledocholithiasis has been rarely reported as a cause of *Clostridium perfringens* bacteremia[17]. We report an unusual case of *Clostridium perfringens* bacteremia caused by choledocholithiasis in the absence of gallbladder stones and with normal CBD diameter. This case demonstrates the importance of pursuing an extensive differential diagnosis because the identification of the underlying source may be necessary to prevent a fatal outcome. Our patient presented with *clostridium perfringens* bacteremia along with newly elevated liver function tests leading to consideration of biliary source for his bacteremia. This led to further imaging for evaluation of the biliary tract despite normal gallbladder and common bile duct seen on abdominal US and CT scan of the abdomen.

Clostridium perfringens bacteremia is a rare occurrence. We would like to emphasis the importance of discovery of the source of bacteremia and the early administration of antibiotics. It is important to recognize the occurrence of intravascular hemolysis with *Clostridium perfringens* bacteremia. Imaging of the biliary system by MRCP or endoscopic US is needed despite normal ultrasound and CT scan of the abdomen when biliary source of *Clostridium perfringens* bacteremia is suspected.

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CASE REPORT

A *de novo* germline *MLH1* mutation in a Lynch syndrome patient with discordant immunohistochemical and molecular biology test results

Fabrice Airaud, Sébastien Küry, Isabelle Valo, Ingrid Maury, Dominique Bonneau, Olivier Ingster, Stéphane Bezieau

Fabrice Airaud, Sébastien Küry, Ingrid Maury, Stéphane Bezieau, CHU Nantes, Service de Génétique Médicale, 9 quai Moncousu, 44093 Nantes Cedex 1, France
Isabelle Valo, Institut de Cancérologie de l'Ouest, Service d' Anatomie et Cytologie Pathologique, 2 rue Moll, 49933 Angers Cedex 9, France
Dominique Bonneau, Olivier Ingster, CHU Angers, Service de Génétique Médicale, 4 rue Larrey, 49933 Angers, France
Author contributions: Airaud F, Küry S and Bezieau S designed research and wrote the paper; Valo I performed the anatomopathology research; Maury I performed the molecular biology research; and Bonneau D and Ingster O performed the clinical investigation.
Correspondence to: Fabrice Airaud, MSc, CHU Nantes, Service de Génétique Médicale, 9 quai Moncousu, 44093 Nantes Cedex 1, France. fabrice.airaud@chu-nantes.fr
Telephone: +33-240-084299 Fax: +33-240-084026
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Abstract

We describe a patient with a *Homo sapiens* mutL homolog 1 (*MLH1*)-associated Lynch syndrome with previous diagnoses of two distinct primary cancers: a sigmoid colon cancer at the age of 39 years, and a right colon cancer at the age of 50 years. The mutation identified in his blood and buccal cells, c.1771delG, p.Asp591Ilefs*25, appears to be a *de novo* event, as it was not transmitted by either of his parents. This type of *de novo* event is rare in *MLH1* as only three cases have been reported in the literature so far. Furthermore, the discordant results observed between replication error phenotyping and immunohistochemistry highlight the importance of the systematic use of both pre-screening tests in the molecular diagnosis of Lynch syndrome.

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Key words: Lynch syndrome; *Homo sapiens* mutL homolog 1; *De novo* mutation; Replication error phenotype; Immunohistochemistry

Peer reviewers: Dr. Francesco Franceschi, Department of Internal Medicine, Catholic University of Rome, Policlinico A. Gemelli, Largo A. Gemelli, 8, 00168 Rome, Italy; Joerg Trojan, Professor, Department of Medizinische Klinik 1, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany; Peter Laszlo Lakatos, MD, PhD, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, H1083 Budapest, Hungary; Jae J Kim, MD, PhD, Professor, Department of Medicine, Samsung Medical Center, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

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INTRODUCTION

Lynch syndrome or hereditary non-polyposis colorectal cancer syndrome (MIM#120435)[1] is a familial form of cancer mainly involving the colon, rectum or endometrium and, more rarely, the small bowel, urinary tract, ovaries, stomach, brain or skin. The pathology is transmitted in an autosomal dominant mode of inheritance with an incomplete penetrance. It is linked to mutations in the genes of the DNA mismatch repair system (MMR), including *Homo sapiens* mutL homolog 1 (*MLH1*), *Homo sapiens* mutS homolog 2 (*MSH2*), and to a lesser extent,

Homo sapiens mutS homolog 6 (*MSH6*) and PostMeiotic Segregation increased 2 (*PMS2*). International scientific community defined the clinical and familial criteria which had to be fulfilled to consider a likely diagnosis of Lynch syndrome, and thereby the value of a mutation screening of *MMR* genes in a patient. The initial recommendations following the Amsterdam (1991)[2] and Amsterdam Ⅱ (1999) criteria[3] were too restrictive. New criteria were therefore proposed at the Bethesda conference in 2004[4], in order to increase the sensitivity of mutation carrier detection. These recommendations include an age at diagnosis younger than 50 years, or younger than 60 years when the tumor has a microsatellite instability-high phenotype (MSI-high), which is the hallmark of tumors linked to a defective *MMR* gene system[5,6].

Here we report on the unique case of a *de novo* mutation encountered by our Oncogenetic Laboratory through its diagnostic activity in Lynch syndrome. In our molecular diagnostic strategy, replication error (RER) phenotyping is performed systematically before sequencing of the *MMR* genes whenever tumor tissue is available, and this is done concomitantly with immunohistochemistry in order to direct the sequencing as far as possible. Sequencing of either *MLH1*, *MLH2* or both genes is initiated each time a tumor is found to be MSI-high or -low (low microsatellite instability: one system with replication error), even when the immunohistochemistry results are ambiguous or show no extinction of a MMR protein. The sequencing step is also initiated under three specific circumstances: (1) the finding of an microsatellite stable (MSS) phenotype with validated Amsterdam criteria; (2) when no tumor material is available; and (3) in the case of a discrepancy between the immunohistochemistry results and the RER phenotype.

Whenever necessary, DNA samples are sent to another laboratory within the French MMR network (Groupe Génétique et Cancer MMR) for further testing of *MSH6*.

CASE REPORT

The patient is a 54 year-old man who had previously presented two distinct primary cancers, a sigmoid colon cancer at the age of 39 years, and a right colon cancer at the age of 50 years. He is the sixth of a family of 10 siblings, three of whom have had polyps removed: two brothers and one sister, at the ages of 38, 42 and 38 years respectively. A fourth sibling, another of his sisters, was diagnosed with cervical cancer at 45 years of age, but it is not in the tumor spectrum for Lynch syndrome. Among the older generations of the patient's family, the maternal grandfather died of colon cancer at the age of 84 years and the mother's medical records report an ovarian cyst and ovariectomy (Figure 1).

The right colon tumor had been removed by a colectomy covering 30 cm of the right colon and 5 cm of the ileum. The tumor measured 7 cm × 5 cm × 1.5 cm and was located in the caecum, invading the terminal ileum. The pathology examination revealed a grade 3 stage for the poorly differentiated, burgeoning and stenosing adenocarcinoma, and cancer staging was classified as pT-3N0Mx. Immunochemistry and molecular biology analyses were performed on formalin-fixed paraffin-embedded material from this tumor.

The immunohistochemistry analysis for the MMR proteins MLH1, MSH2 and MSH6[7] was performed but did not demonstrate a loss of expression, albeit a very weak expression was detected for MLH1 and MSH2.

RER phenotyping was then performed and showed an instable phenotype for four of the five markers routinely tested in our laboratory (BAT25, BAT26, NR21, NR22 and NR24)[8,9], with stability noted only for the NR24 system. The tumor was thus classified MSI-high, and the patient was considered for mutational analysis of *MMR* genes, according to the Bethesda criteria.

In keeping with routine practice at our laboratory, we started with the search for large rearrangements using a commercial Multiplex Ligation-dependent Probe Amplification (MLPA®) kit for *MLH1* and *MSH2* (P003-B1 kit, MRC-HOLLAND, Amsterdam, The Netherlands)[10]. This revealed the isolated loss of *MLH1* exon 16. In such cases of single exon deletion or duplication, we systematically verify the hybridizing sequence of the MLPA probes to exclude a false result caused by a single nucleotide polymorphism (SNP). However, in the present case, the sequence analysis showed a single nucleotide deletion of a G in position 1771 (NM_000249.3: c.1771delG, p.Asp591Ilefs*25). This mutation had previously been described in a Taiwanese Lynch family[11]. It had been classified as deleterious because of the occurrence of a premature stop codon at position 615, which disrupts the interacting protein domains to *Pms*2 and exonuclease 1[12].

From this point on, we set out to investigate the status of the mutation within the tumor. Finding the mutation in a homozygous or hemizygous state would have meant that the second hit in the carcinogenetic process was a loss of heterozygosity, and that the mutation was the first hit. Unfortunately, the poor quality of the DNA extracted from the Formaldehyde-fixed Paraffin-embedded material did not enable us to get readable sequences. We confirmed the presence of mutation c.1771delG (p.Asp591Ilefs*25) in a second independent sample (DNA extracted from a buccal swab spotted onto a FTA paper). Once the mutation had been confirmed, a presymptomatic test was offered to the patient's family. In this context, we tested both parents but did not identify any parental origin for the mutation as both parents tested negative for the mutation, in both blood and buccal cell samples. We then performed the complete sequencing of *MLH1* to exclude any other mutational event that could have been the true cause of the patient's personal and familial history of cancer, but we did not identify anything else.

These results led us to question the patient's paternity, given that a meta-analysis performed in 2005 reported false paternity in a median of 3.7% of births[13]. Nevertheless, a genetic fingerprinting kit (AmpFℓSTR® SGM

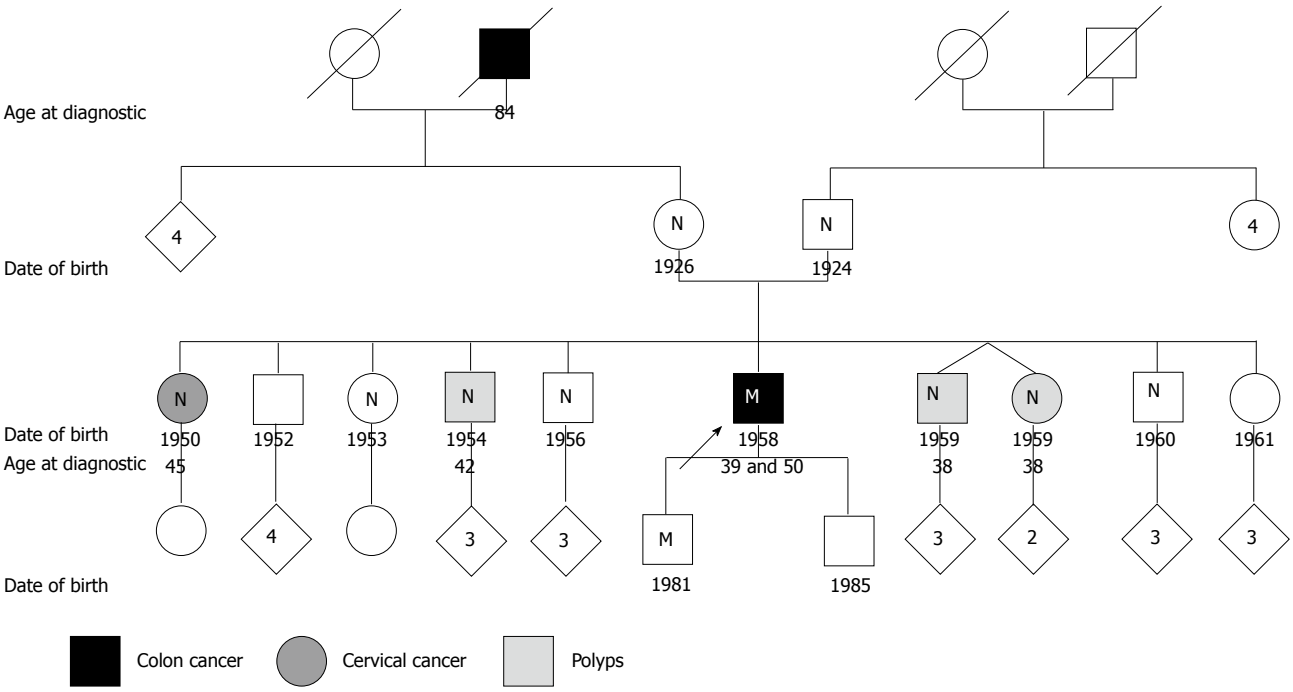


Figure 1 Pedigree. Family tree shows the segregation and clinical manifestations in the family with *de novo Homo sapiens* mutL homolog 1 mutation. N: Normal carrier status; M: Mutated carrier status.

Plus®; Applied) confirmed the paternal and maternal status of the supposed parents without ambiguity.

In order to further investigate the possibility of a mosaicism, we performed a haplotyping study using two frequent SNP within the *MLH1* gene (rs1800734 and rs2241031) chosen for their high heterozygosity frequencies and their belonging to different ancestral haplotypes. However, the lack of proband informativity meant that this analysis did not enable us to identify the maternal or paternal origin of the chromosome harboring the mutation. However, because we had tested two different types of tissues of different origins, i.e., mesodermic for blood cells and ectodermic for buccal cells, we were able to exclude mosaicism with quite a high level of confidence. We were finally able to conclude that the Lynch syndrome-causing mutation observed in our patient was a *de novo* event.

Up to now, we have been able to test seven of the patient's nine siblings and have not identified any of them as a carrier of the mutation, even those who have had polyps or cervical cancer. This could be considered as a further argument for a *de novo* mutation, since it does not follow the Mendelian transmission ratio of 1:2 and it is not concordant with polyp antecedents.

Finally, a presymptomatic test in our patient's 30 year-old son revealed the presence of mutation c.1771delG (p.Asp591Ilefs*25) in a heterozygous state, thereby highlighting the transmission (and conservation) of this *de novo* mutation by the proband.

DISCUSSION

Here we report on a French Lynch family in whom we have identified a frame-shift mutation that induces a premature stop codon in a crucial part of the *MLH1* gene. This mutation has never been reported in the open access *MMR* mutation database (INSIGHT, Newfoundland *etc.*), or in the French MMR network database (unpublished data). Moreover, we have never found this mutation in 592 chromosomes of patients of similar geographic origin who have been tested in our laboratory as part of routine Lynch syndrome screening.

In contrast with certain other genes, such as *NF1,* which exhibit a *de novo* mutation rate of about 50%, this event in *MLH1* is relatively rare (1% to 5%) according to the study recently published by Win *et al*[14]. To our knowledge, a *de novo* point mutation in *MLH1* has only been described three times until now. The first occurrence was a c.2101C>T (p.Gln701X) mutation in exon 18, which was detected in a 35 year-old man[15]. The second was a c.666dupA (p.Asn222Lysfs*4) mutation in exon 8 found in a 31 year-old man[16]. The third mutation was a nonsense mutation in exon 13, c.1459C>T (p.Arg487X) identified in a 36 year-old patient[14]. In all three cases the patients had no family history of colorectal cancer and seemed to develop cancer younger than inherited mutation carriers. In addition to these three single nucleotide mutations, two large deletions have already been published, one of the entire *MLH1* gene and one of exon 15, once again in a young man without any family history of cancer[14,17]. *De novo* mutations seem to be more frequent in *MSH2*, for which four different mutations have already been described[14,18,19], including the recurrent mutation c.942+3A>T. The latter can even be considered as a kind of mutation hotspot, as it has been proved to occur *de novo* with a relatively high frequen-

cy[20]. The nucleotide implicated in this mutation is part of the BAT26 homopolymer containing 26 adenines. This particular context is hypothesized to be responsible for misalignment during replication or recombination. Even though the *de novo MLH1* mutation described here arose in two families of different ethnic origin, a similar explanation cannot be considered.

This case report confirms the relevance of preceding *MMR* gene sequencing by the combination of the two prescreening tests (RER phenotyping and immunohistochmistry) in the molecular diagnostic strategy in Lynch syndrome, especially for young patients without familial antecedents. Indeed, it is worth noting that most of these patients would not have been considered as candidates for mutation analysis according to the Amsterdam I and II criteria; this might be the reason why *de novo* events in *MLH1* were not described prior to the implementation of the Bethesda criteria. It is also interesting to point out the discordance between immunohistochemistry and RER phenotyping results for our patient's tumor, which confirms the benefit of a dual approach for the screening of Lynch syndrome patients. Indeed, MSI-high phenotypes with conservation of protein expression have already been described and can easily be explained when they concern a missense mutation that does not occur in the epitope of the antibody used. Inversely, extinction of a protein associated with an MSS or MSI-low phenotype can also be encountered, especially when the *MSH6* gene is affected[21]. In glioblastoma, in the context of Turcot syndrome, changes in microsatellite profiles have also been described as more subtle than those in colorectal tumors[22] and thus have to be considered very carefully.

In conclusion, the frequency of *de novo* mutations in *MMR* genes may be higher than actually observed in diagnostic laboratories because once a mutation is identified, the parents of the proband are not systematically analyzed in routine practice. This may be because they are deceased, as the average age at molecular diagnosis of our index cases is 53 years, or because they do not wish to be tested

Moreover, we show here that the combined use of molecular biology and immunohistochemistry should be recommended when screening patients with suspected Lynch syndrome. This combined strategy should help to avoid missing a tumor linked to a deficiency in a *MMR* gene, and also to orientate the subsequent sequencing to one of these genes in a more precise and therefore cost-effective manner.

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CASE REPORT

Ischemic colitis and large bowel infarction: A case report

Eugen Florin Georgescu, Doina Carstea, Daniela Dumitrescu, Ramona Teodorescu, Andrei Carstea

Eugen Florin Georgescu, Ramona Teodorescu, Andrei Carstea, Department of Internal Medicine 1/Gastroenterology, Filantropia University Hospital of Craiova, 200136 Craiova, Romania
Doina Carstea, Department of Cardiology, Filantropia University Hospital of Craiova, 200136 Craiova, Romania
Daniela Dumitrescu, Department of Radiology, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Romania
Author contributions: All authors contributed equally to this manuscript.
Correspondence to: Eugen Florin Georgescu, Professor, Department of Internal Medicine 1/Gastroenterology, Filantropia University Hospital of Craiova, 200136 Craiova, Romania. efg@usa.net
Telephone: +40-744782136 Fax: +40-351813110
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Abstract

Ischemic bowel disease results from an acute or chronic drop in the blood supply to the bowel and may have various clinical presentations, such as intestinal angina, ischemic colitis or intestinal infarction. Elderly patients with systemic atherosclerosis who are symptomatic for the disease in two or more vascular beds have multiple comorbidities and are particularly at risk. The clinical evolution and outcome of this disease are difficult to predict because of its pleomorphic aspects and the general lack of statistical data. In this paper, we present the case of a patient who was monitored in our unit for six years. For this patient, we encountered iterative changes in the clinical pattern, beginning with chronic "intestinal angina" and finishing with signs of acute mesenteric ischemia after an episode of ischemic colitis. This evolution is particularly rare in clinical practice, and the case is instructive because it raises discussions about the natural history of the condition and the therapeutic decisions that should be made at every stage of the disease. An important lesson is that ischemic bowel disease should always be considered in patients who have multiple risk factors for atherosclerosis and have experienced recurrent "indistinct" abdominal symptoms. In these cases, aggressive investigation and therapeutic decisions must be taken whenever possible. Despite an absence of standardized protocols, angiographic evaluation and revascularization procedures have beneficial outcomes. Current advances in endovascular therapy, such as percutaneous transluminal angioplasty with stenting, should be increasingly used in patients with chronic mesenteric ischemia. Such therapy can avoid the risks that are associated with open repair. However, technical difficulties, especially in severe stenotic lesions, frequently occur.

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Key words: Ischemic colitis; Intestinal infarction; Mesenteric thrombosis; Acute mesenteric ischemia; Intestinal angina; Mesenteric atherosclerosis

Peer reviewer: Dr. Giuseppe Chiarioni, Gastroenterological Rehabilitation Division of the University of Verona, Valeggio sul Mincio Hospital, Azienda Ospedale di Valeggio s/M, 37067 Valeggio s/M, Italy

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INTRODUCTION

Ischemic colitis (IC) was first described by Boley *et al*[1]. The condition is a common form of ischemic injury to the gastrointestinal tract and represents approximately one half of all cases involving gastrointestinal ischemia[2]. On average, this disease is found in association with approximately 1-3/1000 acute hospital admissions, but occasionally a mild and transient clinical course may lead to misdiagnoses. Elderly patients are typically affected, and the most common admission signs are hematochezia, abdominal pain and diarrhea[3]. The rapid onset of abdominal pain, tenderness over the affected

bowel area (typically the left side of the colon) and mild to moderate hematochezia are the classic signs, although confusion can appear in cases that are considered to be inflammatory bowel disease or various forms of common bacterial colitis[4]. There is a lack of data regarding the natural history and outcomes of IC. A recent study[5] found that IC is clinically presumed in only 24.2% of cases, and unfavorable outcomes (as defined by mortality and/or the need for surgery) occur at a rate of 12.9%, including an overall mortality rate of 7.7%.

The taxonomy of the disease is somewhat unclear, especially when "puzzling" terms, such as ischemic colitis and colonic ischemia, are employed. According to the American Gastroenterological Association (AGA) technical review on intestinal ischemia[6], ischemic bowel disease can be either acute or chronic. Whereas the acute condition includes various forms of acute mesenteric ischemia (AMI) up to intestinal infarction, the milder chronic variant includes chronic mesenteric ischemia (CMI) (also known as "intestinal angina") and colonic ischemia (CI). CI can appear under various clinical-endoscopic aspects, including (1) reversible colopathy (submucosal or intramural hemorrhage); (2) transient colitis; (3) chronic colitis; (4) stricture; (5) gangrene; and (6) fulminant universal colitis. Most of these aspects have a mixed, ischemic-inflammatory pattern in which ischemic colitis results from an inadequate perfusion that leads to potentially life-threatening colonic inflammation[7]. In its chronic forms, healing is associated with a degree of fibrosis that determines colonic stenosis.

Beyond this almost scholastic classification, it is important to bear in mind that in current practice, these three major categories (AMI, CMI and CI) may be quite intricate. Moving from one to another occurs in a mix of clinical presentations that can appear in the same patient at various moments, depending on factors such as localization, extent of vascular disease, severity of ischemia, or bowel distension, among others. Our case reflects such a patient, in whom the clinical pattern changed several times in a couple of years. The case began as chronic "intestinal angina" and finished with signs of acute mesenteric ischemia after an episode of ischemic colitis.

CASE REPORT

We present the case of a male Caucasian patient who was monitored in our unit between 2006 and 2012. The hallmark of his long medical background was a severe vascular pathology that was facilitated by the combination of several risk factors: heavy smoking since the age of 18 (more than 20 cigarettes/d), type 2 diabetes that was poorly controlled by diet and medication for more than 15 years, obesity, dyslipidemia and moderate to severe hypertension since 1993. An episode of acute thrombosis of the left femoral artery occurred in 1999, which resulted in the amputation of his left thigh. The patient continued to smoke and neglect the recommended therapy, and four years later, he suffered another acute thrombotic episode, this time of the right lower limb with the occlusion of the popliteal artery. His right foot was amputated just below the knee. A complete hemostasis investigation was performed at that time, and there was no sign of coagulation abnormality (including less common genetic defects, such as deficiencies of protein C/S, antithrombin Ⅲ, or factor V Leiden mutation). Aspirin and oral anticoagulation by coumadin were strongly recommended, but the patient appeared to disregard the advice by taking his treatment erratically. Finally, in 2005, a coronarography was performed to investigate an episode of unstable angina. The procedure showed a narrow stenosis (75%) of the circumflex artery; a stent (3.5 × 30 mm) was placed after the dilatation with a balloon catheter.

The patient was first hospitalized in the gastroenterology unit in September 2006, when he was 61 years old. The patient complained of postprandial pain that occurred especially after taking his medication, which consisted of beta blockers, thiazides, angiotensin enzyme inhibitors, aspirin, statins and coumadin. Gastroduodenal, biliary, pancreatic and colonic pathologies were eliminated by upper and lower digestive endoscopy, echography and computed tomography (CT) scans. The initial diagnosis was irritable bowel disease, but a lack of response to specific medication, the suggestive medical history and an improvement in the abdominal pain with sublingual nitroglycerin led to the suspicion of intestinal angina. A mesenteric arteriography was performed in January 2007, which showed a severe (> 75%) incomplete thrombotic occlusion of the upper mesenteric artery and a weak perfusion of the affected bowel loop (Figure 1). The case was concluded to be chronic mesenteric ischemia, but endovascular mesenteric revascularization or surgical bypass were not performed because of the patient's lack of compliance. The patient was discharged with a prescription of long-acting nitrates, and pentoxyfillin was added to his antihypertensive, antiagregant and coumadin treatments.

The evolution was more or less stable until March 2011, when the patient was re-admitted to the unit with episodes of persistent diarrhea and hematochezia, which were spaced by sub-occlusive events of colicky left-lower-quadrant abdominal pain and distension, vomiting and vasovagal symptoms. The examination showed mild pyrexia, tachycardia and a distended abdomen, and there was pain and tenderness in the left lower quadrant upon palpation. A digital rectal examination confirmed hematochezia but did not detect any other lesions. Laboratory investigations showed mild anemia (hemoglobin = 11.1 g/dL), neutrophil leukocytosis (14.800 leukocytes/mm^3 with 78% neutrophils), increased values of ESR (130 mm/h), hyperfibrinogenemia (780 mg/dL) and elevated C-reactive protein titers (27 mg/L). Other biochemical tests were within normal ranges. A plain abdominal radiography did not detect pneumoperitoneum or air-fluid levels. The recent use of antibiotics was denied by the patient, and stool cultures for aerobic/anaerobic pathogens (*Clostridium difficile* included) were negative. Amoebiasis serology,

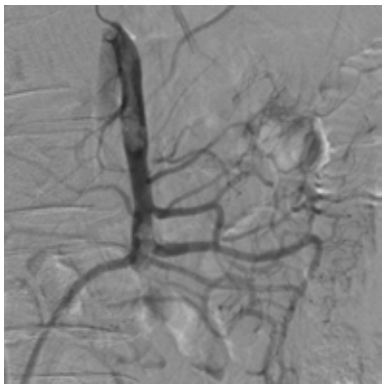


Figure 1 Selective upper mesenteric artery angiogram showing severe incomplete thrombosis with more than a 75% reduction of the arterial lumen and poor perfusion of the bowel loop.

a parasitological investigation and tuberculin intradermoreaction were also negative.

A contrast-enhanced CT scan (Figure 2) revealed segmental colitis involving the splenic flexure and the descending colon. The wall of the colon was markedly thickened with homogeneous enhancement and sharp definition, and it had a "dry" appearance. Concentric layers of low and high attenuation of the colonic wall ("double halo" sign) could be observed on sagittal sections, which suggested colonic edema. A long and narrow area of axial stenosis was present just below the splenic flexure. The stenosis extended to the distal third of the descending colon. Cecal and right colic distension were also observed, but there were no pericolic streakiness or fluid collections.

A colonoscopy found segmental edematous and hemorrhagic areas of the colonic mucosa surrounding large and deep ulcerations that were covered by pseudomembranes. When the pseudomembrane was washed off, the ulcerations revealed an erythematous and congestive granulation tissue. "Geographic"-like areas of mucosal denudation and "cobble stoning" were also observed (Figure 3A). The rectum was spared by an abrupt transition between normal and affected mucosa, and the ulcerative lesions extended to the sigmoid and descending colon. A narrow, long axial inflammatory stenosis was observed just below the splenic flexure. Biopsies showed edema, submucosal hemorrhage and necrotic areas, and an inflammatory infiltration with intravascular thrombi. The crypts had an atrophic appearance, but no cryptitis/cryptic abscesses or granulomas were observed (Figure 3B). Hemosiderin-laden macrophages were present in the mucosa and submucosa, and hyalinization and hemorrhages were observed in the lamina propria, which histologically confirmed ischemic colitis.

The therapeutic decision was to pursue a conservative treatment. After one week of bowel rest, fluid replacement, broad spectrum antibiotics and parenteral nutrition, the patient recovered well and was discharged. Six months later, in November 2011, the patient returned to the emergency room with acute abdominal pain, sudden evacuation of his bowel contents, abdominal distention,

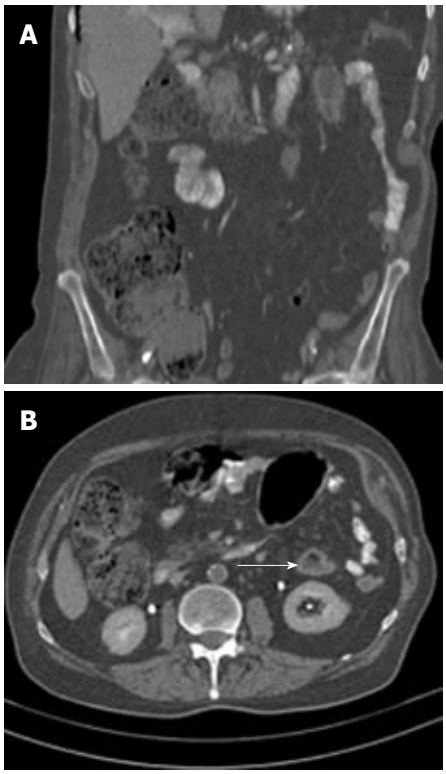


Figure 2 Contrast-enhanced abdominal computed tomography scans suggesting segmental colitis involving the splenic flexure and the descending colon. A: Coronal sections with zones of mural thickening of the colon just below the splenic flexure and a long and narrow axial stenosis of the descending colon; B: Sagittal sections showing (arrow) the "double halo" sign, a tomographic equivalent of the classic "thumbprinting" that is observed in barium enemas.

fever, vomiting and gross hematochezia. Abdominal tenderness, rebound and guarding occurred rapidly with tachycardia, polypnea and hypotension. A CT scan showed a huge thrombus in the abdominal aorta, which extended below the first lumbar vertebra and occluded more than 95% of the aortic lumen (Figure 4). The thrombosis spared the emergence of the celiac trunk and the upper mesenteric artery and appeared to completely occlude the origin of the inferior mesenteric; in the clinical context, this outcome suggested an intestinal infarction. An emergency laparotomy was performed in the following hours. The laparotomy showed a segmental infarction of the descending and sigmoid colon with a transmural hemorrhage and necrosis. Similar lesions were identified at the hepatic flexure. The attached mesentery was also hemorrhagic, and marked colic distention was observed. A total colectomy with ileostomy was successfully performed *per primam*, but unfortunately, the patient was lost in the following days after sepsis and cardiovascular complications.

DISCUSSION

This instructive case presented several clinical aspects of atherothrombotic disease of the mesenteric territory. Most surveys do not include mesenteric ischemia among the major clinical consequences of atherosclerosis, especially considering that coronary and cerebrovascular

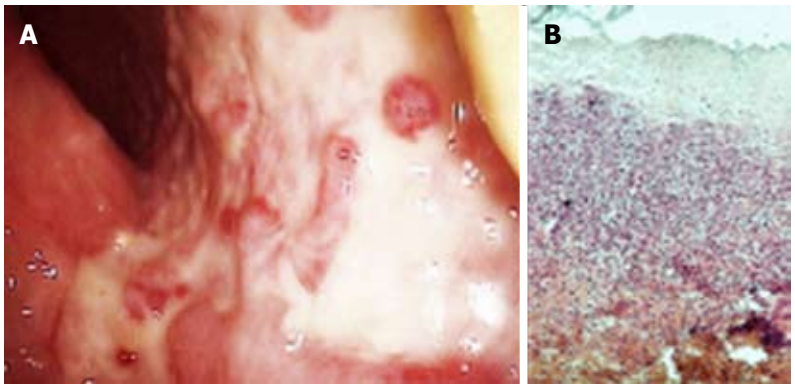


Figure 3 Morphologic changes confirming ischemic colitis by lower digestive endoscopy and histology. A: Areas of mucosal ulceration, pseudomembranes and subsequent granulation observed at colonoscopy; B: Biopsy specimens from affected areas showing submucosal necrosis, inflammatory infiltration, cryptal atrophy and hyalinization. Hematoxylin-eosin staining, ×100. Courtesy Dr. Simionescu.

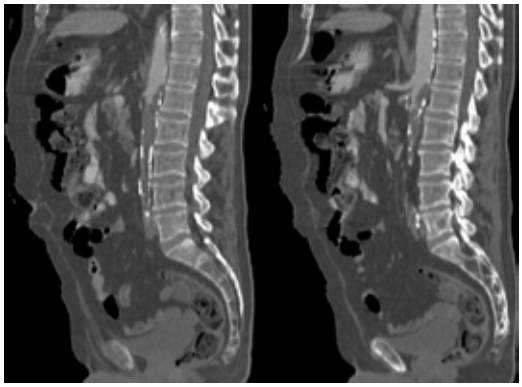


Figure 4 Calcification of the abdominal aorta with extensive thrombosis inside, which completely obstructed the lumen below the 2nd lumbar vertebra. Note that the celiac trunk and upper mesenteric artery are spared, whereas the lower mesenteric is completely occluded.

events with peripheral arterial disease may be credited with 60%-75% of the clinical manifestations of the disease[8-10]. However, even if mesenteric ischemia is not listed in those studies, there is a significant degree of overlap, as 41% of patients present symptoms of the disease in two or more vascular beds[11]. Moreover, recent research[12] found a higher incidence than was preliminarily reported[13,14], increasing the estimate of hospitalizations to 16.4/100 000. Multi-vascular disease is encountered in 24% of cases, and comorbidities, such as hypertension (72%), diabetes (21%) and coronary artery disease (21%), frequently occur[12]. This observation is consistent with our case. In addition to mesenteric arterial thrombosis and embolization, other medical conditions can cause bowel ischemia, such as mesenteric venous thrombosis, trauma, small vessel disease (i.e., diabetes mellitus, vasculitis, amyloidosis, rheumatoid arthritis, radiation), hematologic disorders (i.e., protein C/S or antithrombin Ⅲ deficiency, sickle cell disease), shock, medications (i.e., digitalis, diuretics, nonsteroidal antiinflammatory drugs (NSAIDs), catecholamines, estrogens, danazol, neuroleptics), colonic obstruction, cocaine abuse or long-distance running[15]. In our case, iterative mesentero-aortic thrombosis was the primary cause of the clinical manifestations of the ischemic bowel disease that was encountered in our patient; however, other secondary factors, such as diabetic vasculopathy and even chronic obstructive pulmonary disease[16], should not be overlooked.

Clinical patterns of mesenteric ischemia may be either acute ("gangrenous") or chronic ("nongangrenous") forms[15,17]. Nongangrenous forms (80%-85%) are typically transient and reversible but can progress to chronic and irreversible strictures (10%-15%) or chronic segmental colitis (20%-25%). Acute forms[18] have four major causes: sudden complete arterial occlusion by emboli (50%), thrombosis of atherosclerotic stenosis (20%), small vessel occlusion (20%) and venous thrombosis (10%). Whereas embolic occlusion of the mesenteric arteries is typically brutal and dramatic, often presenting the hallmark "pain out of proportion with physical findings", acute thrombosis of a stenotic atherosclerotic lesion may have a more insidious onset, and there may be a history of intestinal angina, which was the case in our patient. It is important not to miss the clinical signs of chronic mesenteric ischemia in a vascular patient, and it is also important to use angiography to aggressively evaluate the arterial damage in the mesenteric territory. Following the individual patient's anatomic and comorbidity considerations, early revascularization with percutaneous angioplasty/stenting or open repair may prevent a worse outcome, although protocols are not yet fully standardized, and symptomatic recurrences that require reinterventions occur as frequently as 20% of the time[19].

Chronic nongangrenous colonic ischemia, which is frequently expressed by ischemic colitis, indicates a higher degree of impairment of the intestinal blood flow and, as a consequence, more severe vascular damage. The colon is particularly susceptible to hypoperfusion, and low-flow states often precipitate to preexisting mesenteric microvascular atherosclerosis. "Watershed areas", such as the splenic flexure (Griffith's point), ileocecal junction and rectosigmoid, are specific localizations of ischemic lesions, but diffuse and extended ischemic injury of the entire bowel can also be encountered[20]. Ischemia is segmental and superficial, and it only extends to the mucosa and submucosa ("partial mural ischemia")[20,21]. The subsequent inflammation and fibrosis that follow this decrease in the blood supply lead to a "true" colitis that includes edema, neutrophil afflux and hyalinization. Ghost cells and hemosiderin-laden macrophages are highly specific findings that can facilitate

diagnosis; these findings should be differentiated from other entities, such as infectious colitis (i.e., *Salmonella*, *Shigella*, *Campylobacter*, *E. coli* O157:H7, *Clostridium difficile*), inflammatory bowel disease, medication-induced colitis (i.e., NSAIDs, hormones, anticoagulants, diuretics, antibiotics), malignancy, fecal impaction with stercoral ulcer, systemic disorders and amyloidosis[22]. Although therapy classically relies on conservative treatment and anticoagulation, the recurrence of disease, especially in high-risk vascular patients, can impose angiographic evaluation and revascularization procedures that may have beneficial outcomes despite an absence of standardized protocols[22,23].

In conclusion, ischemic bowel disease may be an expression of systemic atherosclerosis, especially in patients who have cumulative risk factors, such as dyslipidemia, diabetes or smoking, and a multi-vascular atherothrombotic pathology. These patients occasionally present all of the clinical forms of mesenteric ischemia, including intestinal angina, ischemic colitis and intestinal infarction. The symptoms may develop over several years and may be intricate, so their recognition is important. Simple anticoagulation does not always prevent major incidents, and revascularization procedures, such as percutaneous angioplasty or stenting, may ensure a better prognosis. In patients with ischemic bowel disease, the important lesson is to always consider aggressive investigations and therapeutic decisions while taking into account the associated benefits and risks. Angiographic evaluation and revascularization procedures are associated with beneficial outcomes despite an absence of standardized protocols. Current advances in endovascular therapy, such as percutaneous transluminal angioplasty with stenting, should be increasingly used in patients with chronic mesenteric ischemia. These procedures will limit the risks that are associated with open repair. However, technical difficulties, such as undistensible stenotic lesions, frequently occur.

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CASE REPORT

Gastrointestinal stromal tumor presenting with prominent calcification

Naoki Izawa, Takeshi Sawada, Ryuichi Abiko, Daisuke Kumon, Mami Hirakawa, Mika Kobayashi, Nobuyuki Obinata, Masahito Nomoto, Tadateru Maehata, Shun-ichi Yamauchi, Takefumi Kouro, Takashi Tsuda, Satoshi Kitajima, Hiroshi Yasuda, Keiichi Tanaka, Ichiro Tanaka, Masahiro Hoshikawa, Masayuki Takagi, Fumio Itoh

Naoki Izawa, Takeshi Sawada, Ryuichi Abiko, Daisuke Kumon, Mami Hirakawa, Mika Kobayashi, Nobuyuki Obinata, Masahito Nomoto, Tadateru Maehata, Shun-ichi Yamauchi, Takefumi Kouro, Takashi Tsuda, Satoshi Kitajima, Hiroshi Yasuda, Fumio Itoh, Division of Gastroenterology and Hepatology, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan
Keiichi Tanaka, Ichiro Tanaka, Division of Gastroenterological and General Surgery, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan
Masahiro Hoshikawa, Masayuki Takagi, Department of Pathology, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan
Author contributions: Izawa N and Sawada T contributed equally to this work; Izawa N and Sawada T wrote the manuscript; Abiko R, Kumon D, Hirakawa M, Kobayashi M, Obinata N, Nomoto M, Maehata T, Yamauchi S, Kouro T, Tsuda T, Kitajima S, Yasuda H, Tanaka K and Tanaka I provided medical care; Hoshikawa M and Takagi M reported pathological findings; and Itoh F gave final approval for publication.
Correspondence to: Takeshi Sawada, MD, PhD, Division of Gastroenterology and Hepatology, St. Marianna University School of Medicine, 2-16-1 Sugao Miyamae-ku, Kawasaki, 216-8511, Japan. t2sawada@marianna-u.ac.jp
Telephone: +81-44-9778111 Fax: +81-44-9765805
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Abstract

We present a rare case of a gastrointestinal stromal tumor (GIST) in the stomach with prominent calcification at presentation. A 61-year-old woman visited our hospital because of epigastric discomfort. A spherical calcified lesion with a diameter of about 30 mm was incidentally shown in the left upper quadrant on an abdominal X-ray. Computed tomography demonstrated that the tumor was growing from the upper gastric body, with calcification in the peripheral ring area. A laparoscopic partial gastrectomy was performed, and the resected specimen revealed a well-circumscribed tumor with exophytic growth from the gastric muscularis propria. Microscopic examination revealed spindle-shaped tumor cells with calcification and hemorrhage. Additionally, positive immunoreactivity of the tumor to KIT and CD34 and a low mitotic index resulted in the diagnosis of very low risk GIST. There are a few case reports of heavily calcified GIST, although solitary or punctate calcification of primary GIST has been reported in several case series. Dystrophic calcification of necrotic or degenerative tissue is the supposed cause of primary calcified GISTs. In contrast, appearance of calcification after administration of imatinib mesylate, which may be one indicator of disease response, is possibly caused by a different mechanism.

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Key words: Gastrointestinal stromal tumor; Calcification; Stomach; Computed tomography; Imatinib mesylate

Peer reviewer: Dr. Xiao-Yun Liao, Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Room JF-208E, Boston, MA 02215, United States

Izawa N, Sawada T, Abiko R, Kumon D, Hirakawa M, Kobayashi M, Obinata N, Nomoto M, Maehata T, Yamauchi S, Kouro T, Tsuda T, Kitajima S, Yasuda H, Tanaka K, Tanaka I, Hoshikawa M, Takagi M, Itoh F. Gastrointestinal stromal tumor presenting with prominent calcification. *World J Gastroenterol* 2012; 18(39): 5645-5648 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i39/5645.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i39.5645

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common primary mesenchymal neoplasms of the gastrointestinal tract[1,2]. In the past decade, histological and clinical features of GISTs have become increasingly clear[1,2], however, calcified tumor mass is an unusual feature of GISTs at initial presentation, and the mechanism is not clear. We describe here an uncommon case of gastric GIST with prominent calcification.

CASE REPORT

A 61-year-old woman without a significant medical history visited our hospital due to epigastric discomfort. There were no positive findings on physical examination. Initial laboratory tests demonstrated low hemoglobin level (9.6 g/dL), which was probably due to iron deficiency anemia.

An abdominal X-ray showed a spherical calcified lesion measuring approximately 30 mm in the left upper quadrant (Figure 1A). An upper gastrointestinal barium study revealed an exogastric mass (Figure 1B). A plain computed tomography (CT) demonstrated calcification in the peripheral ring area of the tumor growing from the upper gastric body. With an abdominal enhanced CT, the tumor was enhanced heterogeneously, except for the calcified section (Figure 2A and B). There was no evidence of liver metastasis or diffuse peritoneal spread. Magnetic resonance imaging of the central portion of the tumor showed low signal intensity on T1-weighted images (Figure 2C) and high signal intensity on T2-weighted images (Figure 2D). Indicative of calcification, peripheral portions showed low intensity on both T1 and T2-weighted images. Endoscopic examination revealed a round submucosal tumor with the upper part depressed (Figure 3A). Endoscopic ultrasonography (EUS) indicated the tumor originated from the fourth layer of the gastric wall (Figure 3B). Although EUS-guided fine needle aspiration was considered for the diagnosis of the submucosal lesion, it was impossible to perform due to calcification of the tumor wall with a sonic shadow (Figure 3B). After informed consent was obtained, laparoscopic partial gastric resection was performed.

The resected specimen revealed a well-circumscribed tumor measuring 32 mm × 23 mm × 23 mm originating from the gastric muscularis propria. The sliced surface of the tumor showed a firm, solid, whitish-gray parenchyma with circular calcification and internal bleeding (Figure 4A). Microscopic investigation revealed spindle-shaped tumor cells (Figure 4B) with calcification and hemorrhage. The cells exhibited low mitotic activity of fewer than 5 mitoses per 50 high power field (HPF) and no prominent signs of nuclear atypia. Immunohistochemical staining demonstrated positive reactivity to KIT (Figure 4C) and CD34 (Figure 4D). On the other hand, there was negative reactivity to S-100 protein, smooth muscle actin, and desmin (not shown). Since the mitotic index of this tumor was less than 5/50 HPF, its diagnosis was established as very low risk GIST according to risk stratification guidelines[1]. The patient had an uneventful postoperative course and was discharged on the 7th postoperative day. The patient has been followed up for 10 mo without any signs of recurrence.

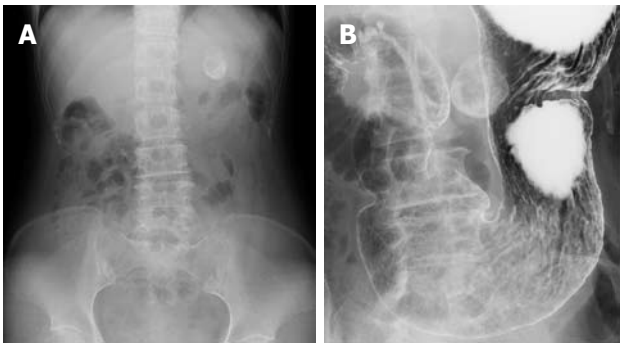


Figure 1 Radiological findings. A: Abdominal X-ray indicated a spherical calcified lesion in the left upper quadrant; B: Barium study showed a round calcification bordering the gastric wall.

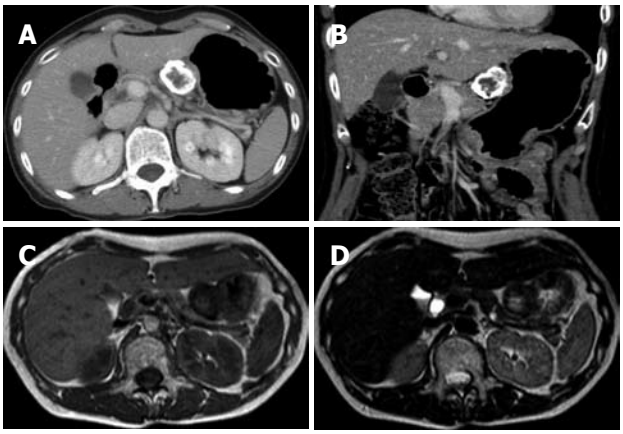


Figure 2 Computed tomography and magnetic resonance imaging findings. Contrast-enhanced axial (A) and coronal (B) computed tomography examination demonstrated that the marginal zone of the tumor was calcified, and that the internal portion of the tumor was enhanced heterogeneously. Magnetic resonance imaging T1-weighted image (C) and T2-weighted image (D) revealed a low intensity marginal zone of tumor reflecting calcification.

DISCUSSION

GISTs are defined as mesenchymal tumors in the gastrointestinal tract that express KIT, a tyrosine kinase growth factor receptor[1]. Clinical, histological and molecular features of GISTs have become increasingly clear in the past decade[1,2], and radiologic appearances of these tumors have also been well described[2-7]. Small GISTs (< 5 cm) are often seen as well-defined with a homogeneous appearance on contrast-enhanced scans, whereas larger lesions (> 10 cm) are more likely to have irregular and lobulated margins with heterogeneous enhancement[2-4].

Although several cases of primary GIST with calcification have been reported[3-6], solitary or punctate calcification was shown in most cases[4,5]. In contrast, there are only six case reports describing heavily calcified

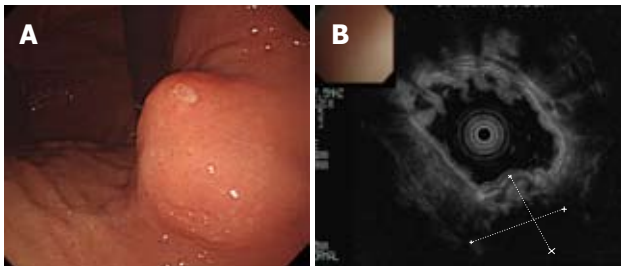


Figure 3 Endoscopy and endoscopic ultrasound of stomach. A: Endoscopic examination revealed a round submucosal tumor; B: The tumor originated from the fourth layer of the gastric wall as indicated by endoscopic ultrasound. The deeper section could not be visualized because of calcification.

GISTs[8-13]; three cases located in the stomach, one in the colon, and two in the rectum. Although Ghanem *et al*[4] referred to a solitary calcification as an aggressive finding, the evidence was not clearly shown. Regarding primary lesion, Tateishi *et al*[5] suggested that the frequency of calcification was not a reliable finding for the distinction of low- and high-grade GISTs. Particularly, with respect to malignant GIST of the stomach, no CT feature (including calcification) other than size was found to have predictive value[6].

The mechanism of calcification at presentation is unclear. Yoshida *et al*[8] suggested that the calcification of GIST may be developed mostly from necrotic tissue. In the earlier literature, a true GIST would be included in the imaging studies of leiomyoma, leiomyoblastoma, or leiomyosarcoma arising in the gastrointestinal tract. Prior to the appearance of the definition of GIST, the major mechanism of calcified gastric submucosal tumors was also supposed to be dystrophic calcification of necrotic or degenerative tissue[14].

In addition, several cases have demonstrated increasing calcification in mesenteric metastasis after therapy with imatinib mesylate[7,15]. Histological examination of liver metastasis after therapy showed myxomatous degeneration, leaving small pyknotic nuclei in an eosinophilic myxoid background, with no signs of an inflammatory reaction or necrosis[16,17]. Imatinib mesylate alters the balance from cellular proliferation to apoptosis by inhibiting the tyrosine kinase activity of KIT[16]. Because cellular death of GISTs following imatinib therapy is by apoptosis and not by necrosis, which is the principal mechanism of cell death following conventional cytotoxic chemotherapy[7], the mechanism of calcification following administration of imatinib is probably different from that of primary calcification.

[^{18}F]-fluorodeoxyglucose-positron emission tomography has been reported as useful for the evaluation of early response to imatinib mesylate treatment[16,18,19]. Furthermore, a decrease in tumor size or a tumor density on post-treatment CT has been reported as a good predictor for tumor response[19,20]. Sandrasegaran *et al*[7] also reported that four of twenty-two mesenteric masses showed calcification as well as cystic changes following at least 6 mo of therapy. It is suggested that calcification, in addition to a reduction in tumor size and extensive cystic changes, may indicate disease response[15].

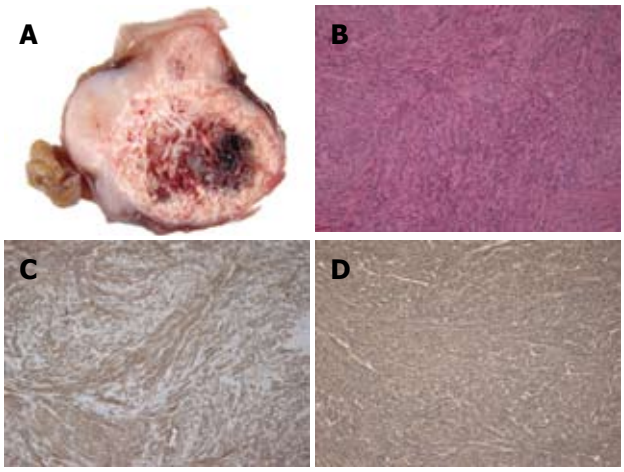


Figure 4 Pathological findings. A: Sliced sections of the resected mass demonstrated a firm, solid, whitish-gray parenchyma with circular calcification and internal bleeding; B: Microscopically, the tumor was characterized by spindle-shaped tumor cells (hematoxylin and eosin, original magnification ×100); Immunohistochemically, the tumor cells were positive for KIT (C) and CD34 (D).

In conclusion, we present here a rare case of gastric GIST with prominent calcification. To assess the mechanism and clinical relevance of calcification of GIST at presentation and following administration of imatinib, further accumulation of patients with pathological investigation would be required.

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CASE REPORT

Hepatothorax due to a right diaphragmatic rupture related to duodenal ulcer perforation

Se-Jin Baek, Jin Kim, Sung-Ho Lee

Se-Jin Baek, Jin Kim, Department of Surgery, Korea University Anam Hospital, Seoul 136-705, South Korea
Sung-Ho Lee, Department of Thoracic Surgery, Korea University Anam Hospital, Seoul 136-705, South Korea
Author contributions: Baek SJ contributed to this paper; Kim J and Lee SH provided general review.
Correspondence to: Jin Kim, MD, PhD, Department of Surgery, Korea University Anam Hospital, Inchon-ro 73, Seongbuk-gu, Seoul 136-705, South Korea. mrgs@korea.ac.kr
Telephone: +82-2-9205346 Fax: +82-2-9281631
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Abstract

Here, we present the case of a 53-year-old man with a hepatothorax due to a right diaphragmatic rupture related to duodenal ulcer perforation. On admission, the patient complained of severe acute abdominal pain, with physical examination findings suspicious for a perforated peptic ulcer. Of note, the patient had no history of other medical conditions or recent trauma, and the initial chest radiography and laboratory findings were not specific. A subsequent abdominal computed tomography revealed intrathoracic displacement of the liver, gallbladder, transverse colon and omentum through a right diaphragmatic defect. The patient then underwent an explorative laparotomy that confirmed duodenal ulcer perforation. A primary repair of the duodenal perforation was performed, and the diaphragmatic defect was repaired using a polytetrafluoroethylene patch after the organs were reduced and the cavity irrigated. This particular case proves interesting as right-sided spontaneous diaphragmatic ruptures are very rare and difficult to diagnose. Additionally, the best treatment for such large diaphragmatic defects is still controversial, especially in cases of intrathoracic or intra-abdominal contamination.

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Key words: Diaphragmatic rupture; Hepatothorax; Duodenal ulcer perforation; Polytetrafluoroethylene mesh

Peer reviewers: Dr. Michael Keese, Klinik für Gefässchirurgie, Universität Frankfurtm, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany; Dr. Michael Leitman, Department of Surgery, Beth Israel Medical Center, 10 Union Square East, Suite 2M, New York, NY 10003, United States; Dr. Frank Ivor Tovey, Department of Surgery, University College London, 5 Cossborough Hill, RG214AG Basingstoke, United Kingdom

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INTRODUCTION

Non-traumatic, spontaneous diaphragmatic ruptures are extremely rare, accounting for approximately 1% of all diaphragmatic ruptures. Here, we present the case of an acute right diaphragmatic rupture related to duodenal ulcer perforation which resulted in displacement of the liver, gallbladder, transverse colon and omentum into the intrathoracic cavity.

CASE REPORT

A 53-year-old male patient presented to the emergency room complaining of acute abdominal pain, which reportedly started 30 min previously. Of note, he denied any relevant past medical history, any recent trauma, and any medication use. At the time of admission, the patient continued to complain of severe epigastric pain, and a physical exam revealed a rigid abdomen, with both tenderness and rebound tenderness observed throughout the abdomen. His vital signs were relatively stable, with a blood pressure of 120/80 mmHg, a heart rate of 95 beats

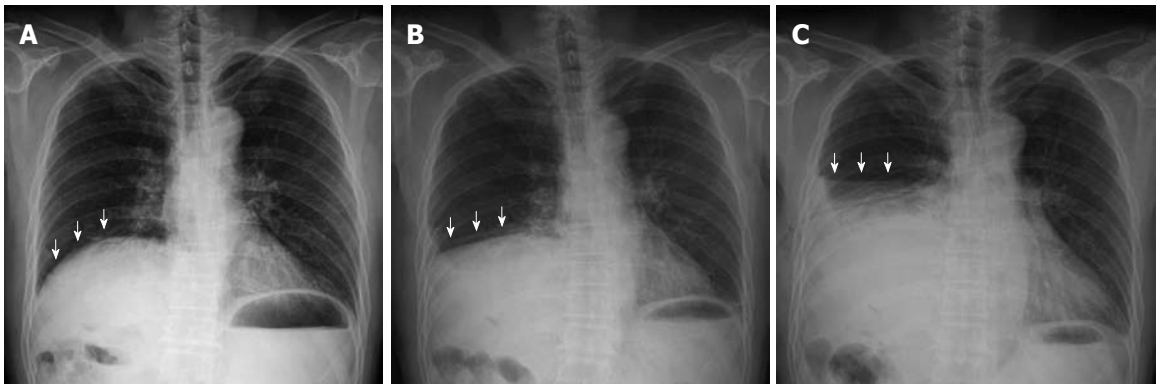


Figure 1 Gradual elevation of the right diaphragmatic border can be seen (arrows). A: Chest radiography on admission; B: Two hours after admission; C: After thoracostomy.



Figure 2 Chest computed tomography coronal reformatted images revealing intrathoracic displacement of the liver, bowel, and omentum through the defect (arrowheads) of the diaphragm (arrows).

per minute, oxygen saturation of 98%, and a body temperature of 37 ℃. The initial chest radiograph revealed a slight elevation of the right diaphragmatic border, though at the time this was not deemed to be significant (Figure 1A). Further laboratory findings showed no other abnormalities, such as anemia or leukocytosis (Figure 1). Due to the generalized peritonitis on the physical exam, a perforated peptic ulcer was suspected, and thus an abdominal computed tomography (CT) was performed. Unexpectedly, no free air or fluid collections were observed in the abdominal cavity. Instead, the CT showed significant displacement of the right lobe of the liver, the bowel and the omentum into the right hemithorax, with the reformatted coronal images providing better views of

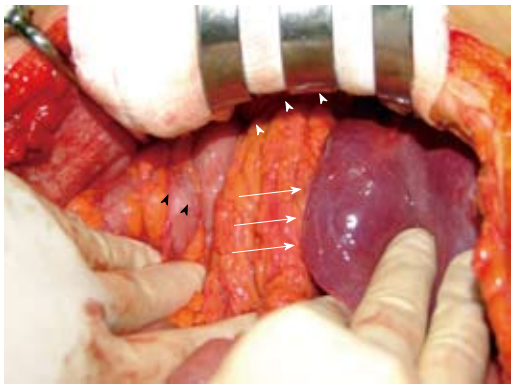


Figure 3 Herniation of the right hepatic lobe, gallbladder, transverse colon, and omentum through the diaphragmatic defect (white arrowheads), showing only the left hepatic lobe (white arrows) and residual transverse colon (black arrowheads) in the right upper abdomen.

the right diaphragmatic border and showing a loss of a part of the diaphragm (Figure 2). Notably, pneumothorax and hydrothorax were also observed, and an injury to the lung parenchyma could not be ruled out.

Initially, a tube thoracostomy was performed, with significant care taken to avoid any injury to the liver and/or bowel. As foul-smelling fluid and bowel contents were noted in the drainage from the chest tube, bowel perforation was also suspected along with diaphragmatic rupture. Accordingly, an exploratory upper midline laparotomy was performed 4 h after admission. At first no evidence of bowel perforation - such as fluid collection or fecal material - was observed in the abdomen. Instead, only the left lobe of the liver was found to have protruded into the right upper abdomen, with the right lobe rotated and herniated into the right hemithorax through the right diaphragmatic defect, along with the gallbladder, transverse colon, and omentum (Figure 3). Exploration of the entire abdomen revealed the site of perforation (approximately 0.5 cm in size) at the first portion of the duodenum immediately distal to the pylorus. Before reducing the herniated organs, a primary repair of the duodenal perforation was performed.

The medial portion of the right side of the diaphragm



Figure 4 Chest radiograph 12 d after surgery showing normal positioning of the right diaphragmatic border compared to the preoperative chest radiograph.

was nearly lost, with only the lateral portion remaining. As this lateral segment blocked reduction, the herniated organs were returned to their respective appropriate locations after the lateral diaphragm was incised, leaving a 12 cm × 10 cm defect. Because of the large size of this defect, primary repair was not possible. Accordingly, another chest tube was inserted just above the diaphragm after the thoracic cavity was thoroughly irrigated. Repair of the diaphragmatic rupture was then performed using a polytetrafluoroethylene patch (GoreTex; W.L. Gore, Flagstaff, AZ, United States) with 1-0 prolene sutures. No other evidence of perforation was noted in the herniated bowel. The abdominal cavity was then irrigated, and an omental patch was applied to the site of the duodenal perforation repair. After surgery, the patient was immediately sent to the intensive care unit for mechanical ventilation, whereby the thoracostomy tubes and abdominal drains were removed in sequence. A post-operative chest radiograph revealed that the right diaphragmatic border had returned to a normal position (Figure 4). After marked improvement in duodenal edema, the patient was discharged on post-operative day 20 with no further complications. Although the patient took oral antibiotics for only one week after discharge, no postoperative complications, including infections, occurred during the following year.

DISCUSSION

Diaphragmatic rupture is a rare complication of abdominal or thoracic trauma, reported in 1%-7% of major blunt trauma patients and 10%-15% of penetrating trauma patients[1,2]. However, approximately 1% of all diaphragmatic ruptures occur spontaneously, often resulting from a sudden increase in abdominal pressure secondary to heavy physical effort, sudden twisting movements, childbirth, and/or severe coughing[3-5]. Other cases have been reported to be caused by static sport activities (e.g., Pilates) or associated with endometriosis[6]. As the patient described here had no recent history of trauma, the exact reason for his diaphragmatic rupture remains unclear. However, given the clinical context, we conjecture that leaked digestive juice may have corroded the diaphragm, as occurs in cases of empyema. Another possibility is that portions of the diaphragm, such as the septum, may have been weakened or altered by previously unrecognized trauma. If this occurred, the pain resulting from the subsequent duodenal ulcer perforation and the associated abdominal muscle tension may have increased the intra-abdominal pressure, thus prompting the weakened diaphragm to rupture. We cannot determine the exact order of these incidents with any certainty. However, for whatever reason, the perforation of the duodenal ulcer seems to have acted as the precipitating event for the diaphragmatic rupture. Roughly 90% of diaphragmatic ruptures occur on the left side[1,2]. Right-sided diaphragmatic ruptures are comparatively rare and difficult to diagnose, as chest radiography often does not reveal any specific signs other than elevation of the right diaphragmatic border. Accordingly, delayed diagnosis is common in right-sided ruptures, often resulting in severe complications, such as strangulation ileus and intrathoracic herniation of the hollow organs (stomach, colon, and small bowel)[2,3].

The initial diagnostic tool is conventional chest radiography, though this modality has limited sensitivity and specificity (17%-40%). Currently, CT is most helpful for emergency diagnosis, as the coronal and sagittal reformatted images offer superior diagnostic value for right diaphragmatic rupture, with a specificity of nearly 100% and a sensitivity of 50%[7,8].

Cases of right diaphragmatic rupture with hepatothorax may result in severe atelectasis of the right lung or tension mediastinum, thereby severely impeding respiration and circulation. As such, if concern exists for right diaphragmatic rupture, an abdominal CT should be performed quickly, and surgical repair via a trans-thoracic or trans-abdominal approach should be considered immediately following radiographic confirmation. Because the size of the defect is often too large for a primary repair to be performed, prosthetic mesh may prove necessary[9,10]. Mesh infection was a significant concern in the case described here, given the intra-abdominal and intra-thoracic contamination from the bowel perforation. Fortunately, such complications did not occur and were likely prevented by the extensive irrigation and intensive antibiotic therapy.

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CASE REPORT

Tracheobronchial nodules and pulmonary infiltrates in a patient with Crohn's disease

De-Gan Lu, Xiao-Qing Ji, Qi Zhao, Cai-Qing Zhang, Zhen-Fang Li

De-Gan Lu, Xiao-Qing Ji, Cai-Qing Zhang, Department of Respiratory Medicine, Shandong Provincial Qianfoshan Hospital, Jinan 250014, Shandong Province, China
Qi Zhao, Zhen-Fang Li, Department of Digestive Medicine, Liaocheng People's Hospital, Liaocheng 252000, Shandong Province, China
Author contributions: Lu DG and Ji XQ treated the patient and collected the data; Lu DG and Ji XQ wrote the paper; Zhao Q, Zhang CQ and Li ZF revised the paper.
Correspondence to: De-Gan Lu, MD, Professor, Department of Respiratory Medicine, Shandong Provincial Qianfoshan Hospital, 16766th Jingshilu, Lixia District, Jinan 250014, Shandong Province, China. deganlu@126.com
Telephone: +86-531-82968368 Fax: +86-531-82963647
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Abstract

Crohn's disease is a granulomatous systemic disorder of unknown etiology. Obvious pulmonary involvement is exceptional. Tracheal involvement in Crohn's disease is even more unusual, only a few cases have been reported to date. We herein report a rare case of tracheobronchial nodules and pulmonary infiltrates in both lungs as a complication of Crohn's disease. A 42-year-old man underwent pancolectomy for multiple broken colon caused by Crohn's disease. Forty days later pulmonary symptoms and radiologic abnormalities were noted. A search for bacterial (including mycobacteria) and fungal in the repeated sputum proved negative. The treatment consisted of intravenous antimicrobials for one month, but there was no improvement in pyrexia or cough and radiologic abnormalities. Fibreoptic bronchoscopy (FOB) was performed and revealed nodes in the trachea and the right upper lobe opening. Histopathology of tracheobronchial nodules and bronchial mucosa biopsy specimen both showed granulomatous inflammation with proliferation of capillaries and inflammatory cells. Oral steroid and salicylazosulfapyridine were commenced and led to marked improvement in symptoms and an almost complete resolution of his chest radiograph. Repeated FOB showed that nodes in the trachea disappeared and the ones in the right upper lobe opening diminished obviously. Crohn's disease can be associated with several respiratory manifestations. The form of tracheal and bronchopulmonary involvement in Crohn's disease is rare and responded well to steroids.

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Key words: Crohn's disease; Inflammatory bowel disease; Lung; Extracolonic involvement

Peer reviewers: Dr. Alan Moss, Department of Gastroenterology, BIDMC/Harvard Medical School, 330 Brookline Ave, Boston, MA 02215, United States; Dr. Adam Cheifetz, Department of Gastroenterology, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215, United States

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INTRODUCTION

Crohn's disease has long been recognized to cause both intestinal and extraintestinal complications. Extra-intestinal manifestations occur in at least 25% of Crohn's disease patients[1]. Although some papers have reported respiratory tract involvement in Crohn's disease, such as tracheobronchitis[2], tracheobronchial stenosis[3], upper bronchial pseudotumors and stenosis[4], granulomatous bronchiolitis[5], reports of involvement in both respiratory tract and lung parenchyma in Crohn's disease are very rare. We describe a patient with Crohn's disease who presented with tracheobronchial nodules and pulmonary infiltrates.

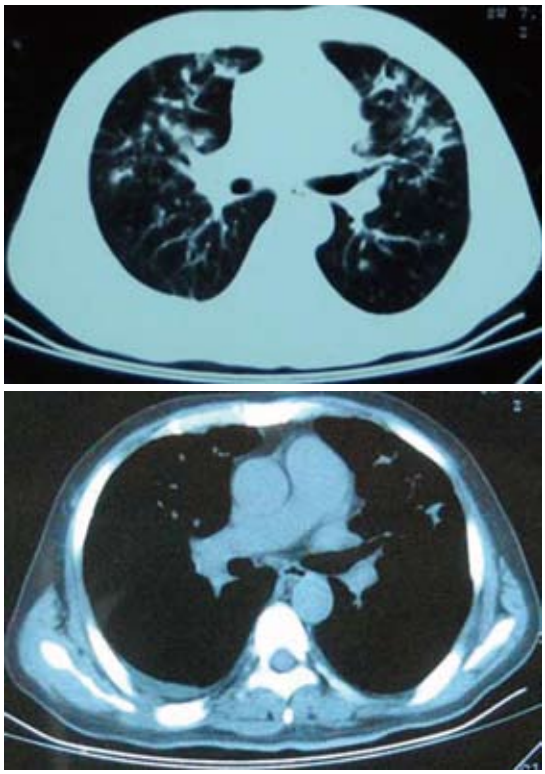


Figure 1 Computed tomography scan of the thorax (January 19, 2009), showing multiple patchy shadows in both lung fields with a little right pleural effusion.



Figure 2 Fibreoptic bronchoscopy (March 9, 2009), showing nodes in the right upper lobe opening.

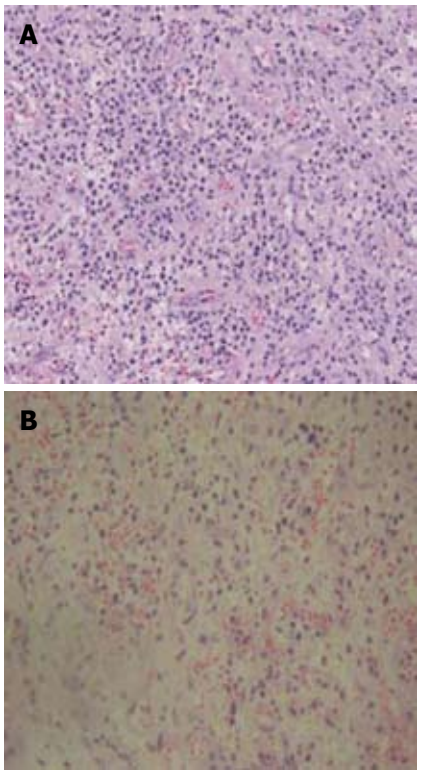


Figure 3 Transfiberoptic bronchoscopy mucosal biopsy, showing polypoid hyperplasia of granulation tissue and hyperplasia of capillary with inflammatory cells (hematoxylin and eosin stain, ×200). A: Tracheobronchial nodules; B: Left lingular lobe.

CASE REPORT

A 42-year-old man was admitted to our department because of a slow onset of cough and pyrexia in January 19, 2009. Forty days prior to this presentation, severe abdominal pain and diarrhea led to his referral to abdominal computed tomography (CT) that revealed free gas under the diaphragm. The patient underwent celiotomy. During operation, multiple broken colon was identified and pancolectomy was performed. The pathology of resected specimen revealed Crohn's disease of colon. His symptoms were resolved after the operation. On admission, he was expectorating about 20 mL of grossly frothy sputum per day and he had a pyrexia of 37.7 ℃. Laboratory data on admission were: white cell counts 8.5 × 10^9/L, 63% segmented neutrophils, a moderate increase of erythrocyte sedimentation rate (30 mm in the first hour). A CT scan of the chest revealed bilateral lung patchy shadows (Figure 1). Initial treatment consisted of intravenous azithromycin (0.5 g, once a day) with aztreonam (2.0 g, twice a day) for 2 wk. A search for bacterial (including mycobacteria) and fungal in the repeated sputum proved negative. The purified protein derivative skin test and cytoplasmic-antineutrophil cytoplasmic antibodies (c-ANCA) were also negative. Intravenous cefoperazone/sulbactam (3.0 g, twice a day) was substituted for 2 wk, but there was no improvement in pyrexia or cough, and his chest CT revealed no dissipation. On day 36 after admission, the patient underwent fibreoptic bronchoscopy (FOB) which showed nodes in the trachea and the right upper lobe opening (Figure 2). Transbronchial lung biopsy was performed in tracheobronchial nodules and left lingular lobe. Histopathology of tracheobronchial nodules and bronchial mucosa biopsy specimen both showed granulomatous inflammation with proliferation of capillaries and inflammatory cells (Figure 3). Fibercoloscope was performed in the following day, which showed cobblestones in the residual sigmoid colon. On day 55, antibiotic treatment was discontinued and the patient was started on prednisone 50 mg daily and salicylazosulfapyridine (SASP) (1.0 g, four times a day) for a presumptive

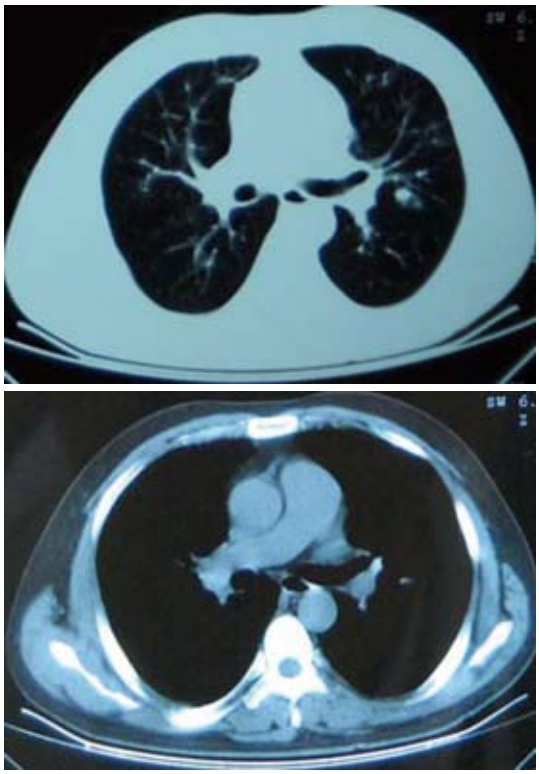


Figure 4 Computed tomography scan of the thorax (April 5, 2009), showing multiple patchy shadows in both lung fields almost disappeared.

diagnosis of Crohn's disease related tracheobronchial nodules and tracheobronchitis associated with pulmonary infiltrates. Within 48 h, the patient's temperature turned normal. There was improvement in cough and the volume of sputum being expectorated a week later. A follow-up CT 3 wk later showed disappearance of the bilateral patchy shadows (Figure 4). He underwent repeated FOB in the following day which showed that nodes in the trachea disappeared and the ones in the right upper lobe opening diminished obviously (Figure 5).

DISCUSSION

Crohn's disease is a granulomatous inflammation of unknown etiology that mainly affects the small bowel, although extracolonic manifestations are common. Extraintestinal manifestations occur in at least 25% of Crohn's disease patients, most commonly in the skin and genitourinary system[6]. It has been rarely reported in the lung. The overall prevalence of concomitant bronchopulmonary manifestations is as low as 0.4%[7]. Several forms of involvement of the lung parenchyma are recognized[8-11]. Diffuse, nongranulomatous interstitial inflammatory lymphoid infiltration has also been described in Crohn's disease[12].

The clinical features of extraintestinal involvement in our patient seem to be interesting for several reasons. Both tracheal-bronchus and lung parenchyma are involved. Transfiberoptic bronchoscopy biopsy of the tracheobronchial nodules and bronchial mucosa in the



Figure 5 Fibreoptic bronchoscopy (April 6, 2009), showing nodes in the right upper lobe opening diminished obviously.

left lingular lobe revealed a benign, nongranulomatous inflammation with proliferation of capillaries and inflammatory cells. Although tracheobronchial involvement in Crohn's disease has been described in literature, the extent of involvement seen in the current patient with nodules in trachea and right upper lobe bronchus, seems quite unusual. There are few previous reports of tracheobronchial nodules. Crohn's disease can involve the lung parenchyma, the tracheobronchial tree, and the pleura[13]. The patient underwent colectomy prior to admission. The pulmonary manifestations of Crohn's disease in some patients may only become clinically remarkable after surgery[14]. Colectomy may aggravate respiratory symptoms[15]. However, the pathogenesis of Crohn's disease-related airway disease remains unknown. It may involve an inflammatory component because a large proportion of these patients are responsive to steroid therapy. The lung and the gastrointestinal tract have a similar embryological origin (a potential source of common antigenicity), and some authors considered a breakdown in the intestinal barrier and entry of dietary antigens as the cause of extracolonic involvement in Crohn's disease[16]. One proposed mechanism is that many of the extraintestinal manifestations of Crohn's disease, including lung disease, are secondary to circulating inflammatory mediators released by the inflamed bowel mucosa[17]. These inflammatory mediators may survive in the pulmonary system for a long time, causing smoldering injuries. This explains the development of pulmonary disease after colectomy in some of the patients with Crohn's disease.

Clinically, when assessing the relationship between respiratory disease and Crohn's disease, one should always keep in mind that the two conditions may have occurred together simply by chance, and therefore other differential diagnoses should be made, such as sarcoidosis, a systemic vasculitis (Wegener's granulomatosis or Churg-Strauss disease), or a prior pulmonary process. In the current patient, the diagnosis of Crohn's disease was initially clearly established on histological grounds of the resected bowel specimen. Although sarcoidosis and Crohn's disease may coexist in the same patient[18], the absence of typical sarcoid granulomata in tracheobron-

chial biopsies, and the lack of evidence for extrapulmonary sarcoid involvement led the authors to rule out sarcoidosis. In view of the absence of histological findings suggestive of Wegener's granulomatosis, the lack of c-ANCA and no extrapulmonary involvement, the diagnosis of Wegener's granulomatosis was unlikely. Post-obstructive pneumonia results from airway obstruction, commonly due to lung cancer. A mismatch in scope of bilateral and diffuse lung infiltrates with tracheobronchial nodules excluded a diagnosis of post-obstructive pneumonia.

The initial lesion of tracheobronchial Crohn's disease seems to be mucosal inflammation, with symptoms of cough and pyrexia[19]. Mucosal inflammation may be accompanied by bronchial suppuration. Biopsy shows either severe nonspecific chronic inflammation or non-caseating tuberculoid granulomas. These appearances have been associated with those in the bowel, and it is possible that the gut and the lung are both affected because they share common antigens. The lung and gastrointestinal tract contain submucosal lymphoid tissues and play crucial roles in host mucosal defense. The similarity in the mucosal immune system causes the same pathogenetic changes. The clinical courses between upper airway and gastrointestinal tract disease are not entirely parallel[20]. It was thought that the tracheobronchial nodules and pulmonary infiltrates were both caused by Crohn's disease, because the pathological finding resembled that of the colon. In addition, they almost vanished as good response to steroids while multiple antibiotic regimens failed to produce clinical improvement.

Drug-induced disease must be kept in mind in patients taking sulfasalazine, mesalamine, methotrexate, and anti-tumor necrosis factor-alpha. Sulfasalazine and 5-aminosalicylic acid derivatives have been reported to cause interstitial pneumonitis, eosinophilic pneumonia, or bronchiolitis obliterans organizing pneumonia in rare cases[21,22]. Other commonly used medications in inflammatory bowel disease patients include 6-mercaptopurine, azathioprine, and methotrexate. These drugs may also cause pneumonia *via* opportunistic infections, or acute pneumonitis or pulmonary fibrosis[23]. Lung abnormalities observed in our patient were unrelated to pharmacological treatment.

Patients with pulmonary involvement of Crohn's disease can be cured with glucocorticoid and SASP, for they are strikingly steroid-responsive frequently. Pulmonary manifestations almost vanished after receiving oral prednisone and SASP in the present case. In all patients with airway disease, marked and long-lasting responses were seen following systemic or inhaled administration of steroids. These findings were not commonly observed for patients with chronic bronchitis without Crohn's disease. Intravenous steroids were required in the initial management of life-threatening complications such as asphyxiating subglottic stenosis or extensive interstitial lung disease. Bronchial lavages with methylprednisolone were effective in some patients with severe airway inflammation. Bronchoscopic dilatation or stent placement should be considered in cases of tracheal stenosis due to Crohn's disease refractory to steroid treatment[24]. Other interventional pulmonology techniques, such as bronchoscopic resection or ablation, could not be generally indicated for treatment modalities potentially contraindicated because of the risk of stenosis or fistula.

In summary, this case illustrates a rare form of tracheal and bronchopulmonary involvement in Crohn's disease with nodes and multiple patchy shadows in both lung fields. As is the case with other forms of pulmonary involvement in Crohn's disease, this manifestation responded well to steroids. It is believed that early investigation and vigorous treatment of bronchopulmonary involvement of Crohn's disease is essential.

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CASE REPORT

Severe acute cholangitis after endoscopic sphincterotomy induced by barium examination: A case report

Zhen-Hai Zhang, Ya-Guang Wu, Cheng-Kun Qin, Zhong-Xue Su, Jian Xu, Guo-Zhe Xian, Shuo-Dong Wu

Zhen-Hai Zhang, Ya-Guang Wu, Cheng-Kun Qin, Zhong-Xue Su, Jian Xu, Guo-Zhe Xian, Department of Hepatobiliary Surgery, Shandong Provincial Hospital Affiliated to Shangdong University, Jinan 250021, Shandong Province, China
Zhen-Hai Zhang, Guo-Zhe Xian, Shuo-Dong Wu, the Second Department of General Surgery, the Second Affiliated Hospital of China Medical University, Shenyang 110004, Liaoning Province, China
Author contributions: Zhang ZH and Wu SD contributed equally to this work; Zhang ZH and Wu SD designed the research; Wu YG and Qin CK discussed the clinical features of the patient; Xu J and Xian GZ analyzed data; Zhang ZH, Wu YG, Qin CK and Su ZX wrote the paper.
Correspondence to: Dr. Shuo-Dong Wu, the Second Department of General Surgery, the Second Affiliated Hospital of China Medical University, No. 36 Sanhao Street, Heping District, Shenyang 110004, Liaoning Province, China. zhangzhenhai410@yahoo.com.cn
Telephone: +86-531-85186363 Fax: +86-531-85186363
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Abstract

Endoscopic sphincterotomy (EST) is considered as a possible etiological factor for severe cholangitis. We herein report a case of severe cholangitis after endoscopic sphincterotomy induced by barium examination. An adult male patient presented with epigastric pain was diagnosed as having choledocholithiasis by ultrasonography. EST was performed and the stone was completely cleaned. Barium examination was done 3 d after EST and severe cholangitis appeared 4 h later. The patient was recovered after treated with tienam for 4 d. Barium examination may induce severe cholangitis in patients after EST, although rare, barium examination should be chosen cautiously. Cautions should be also used when EST is performed in patients younger than 50 years to avoid the damage to the sphincter of Oddi.

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Key words: Endoscopic sphincterotomy; Severe cholangitis; Barium examination; Sphincter of Oddi

Peer reviewers: Dr. Seyfettin Koklu, Department of Gastroenterology, Ankara Education and Research Hospital, 06310 Ankara, Turkey; Atsushi Irisawa, Professor, Department of Gastroenterology, Fukushima Medical University Aizu Medical Center, 10-75 Shiromae, Aizuwakamatsu 960-8555, Japan; Ji Kon Ryu, Professor, Department of Internal Medicine, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seuol 110-744, South Korea

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is an important diagnostic technique for biliary and pancreatic diseases. Endoscopic sphincterotomy has become a well established modality for common bile duct (CBD) stones[1,2]. However, endoscopic sphincterotomy is considered as a possible etiological factor for recurrent ascending cholangitis[3,4]. Here we report a case of severe cholangitis after endoscopic sphincterotomy induced by barium examination, and the patient was recovered after treatment with tienam.

CASE REPORT

A 40-year-old man presented with dull aching epigastric pain for 2 wk. He had a history of CBD stones for 2 years. The patient was averagely built and nourished. No jaundice was present. Biochemical parameters were all

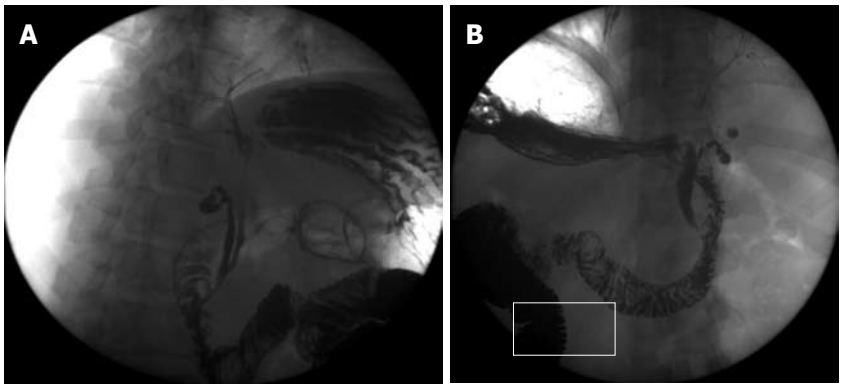


Figure 1 Barium refluxed into the biliary tree of the patient after endoscopic sphincterotomy.

within normal limits. Total white blood cell (WBC) count and hemoglobin measurement were normal. Liver function tests showed that serum bilirubin was 13.5 μmol/L, alkaline phosphatase 55 U/L, aspartate aminotransferase (AST) 40 U/L and alanine aminotransferase (ALT) 35 U/L. Ultrasonography revealed CBD stones in the ampulla of vater and a dilated CBD of 12 mm in diameter. ERCP displayed a dilated CBD. Endoscopic sphincterotomy (EST) was performed in the Second Affiliated Hospital of China Medical University and stones were removed from the biliary tract. The general condition of the patient was good except for slight dull epigastric pain.

To rule out the disease of stomach, barium examination was made 3 d after EST. In the process of barium examination, barium could be seen refluxed into the biliary tree (Figure 1). Four hours later, the patient had a high fever and chills, and the body temperature was climaxed to 39.8 ℃. On investigation, total WBC count was increased to 22.45 × 10^9/L, and hemoglobin was normal. Liver function tests showed serum bilirubin 33.5 μmol/L, alkaline phosphatase 155 U/L, AST 143 U/L and ALT 235 U/L, urine amylase 85 U/L and blood amylase 110 U/L. Ultrasonography showed a dilated CBD and no specific manifestation of the pancreas. Severe cholangitis after endoscopic sphincterotomy induced by barium examination was then diagnosed. The patient was recovered after treated with tienam for 4 d. He is still alive 5 years after the treatment, but suffered from 1-3 recurrent episodes of reflux cholangitis each year.

DISCUSSION

Endoscopic papillotomy with stone extraction continues to be a therapeutic choice and the reference standard in the treatment of symptomatic choledocholithiasis, especially for the solitary bile duct stones up to 12 mm in diameter. The overall success rate with EST was 95.7% in the present report. The most frequent complication encountered was bleeding, followed by acute pancreatitis, acute cholangitis, and perforation[5,6]. It has been found recently that the incidence of acute biliary pancreatitis may be lowered by pancreatic duct stenting[7].

After endoscopic sphincterotomy, the biliary sphincter is rendered permanently insufficient. The loss of this physiologic barrier between duodenum and biliary tract results in duodenocholedochol reflux and bacterial colonization of the biliary tract. Our previous study showed that about 35.9% of the patients with a T-tube after cholecystectomy and choledochotomy had duodenal-biliary reflux. Most of them had hypomotility of the sphincter of Oddi[8]. After EST, biliary reflux of duodenal chyme occurs in most patients, aerobilia is seen in about half, and bacterobilia in all the patients[9,10]. Most patients with bacterobillia did not inevitably develop symptomatic recurrent cholangitis, but 20% patients had upper abdominal pain during the follow-up of about 36 mo[11]. Another study showed that 65% patients after EST had duodenobiliary refux, detected by barium studies, although no clinical symptoms were observed [12]. The presence of bacteria in the biliary system, which is sterile under physiologic conditions, might lead to complications after EST. Misra *et al*[13] studied the incidence of duodenobiliary reflux and acute cholangitis after placement of self-expanding metal stent across the main duodenal papilla, and found that severe reflux of barium was evident in all the patients. However, none of them developed acute cholangitis because of reflux.

To avoid the damage to the biliary sphincter, endoscopic papillary balloon dilation (EPBD) was introduced as a less traumatic alternative to EST in the management of biliary tract stones[14]. However, a high incidence rate of procedure-induced pancreatitis was reported[15]. But, more recently, May *et al*[16] and Mathuna *et al*[17] found that the complication rates of pancreatitis by EPBD were similar to those by EST. Toda *et al*[18] studied the early results of EST and EPBD and found no difference in the early complications between EST and EPBD. The incidence rates of cholangitis by EST and EPBD were 4.0% and 4.2%, respectively. Yasuda *et al*[19] showed that preservation of papillary function after EPBD was not complete, but remained somewhat reduced. Preservation was more successful with EPBD than with EST. The incidence of pneumobilia was significantly higher in post-EST than in post-EPBD patients. Yasuda *et al*[20] also found that during long-term follow-up, patients who underwent endoscopic sphincterotomy (ES) had significantly more biliary complications than those who underwent EPBD. The biliary sphincter dysfunction after ES results in additional late complications.

Reflux of duodenal contents into the biliary tract af-

ter EST was the consequence of reduction or abolition of sphincter activity, as documented by manometry even 15 years following the sphincterotomy[21]. One study found that sphincterotomy was associated with a 5-fold higher incidence of recurrent brown CBD stones compared with choledocholithotomy[22]. One study reported the potentials of endoscopic papillary large balloon dilatation (EPLBD) with minor EST for the complete removal of CBD stones and found that the recurrence of CBD stones was especially low in cases of periampullary diverticulum treated with EPLBD by minor EST[23].

In our patient after EST, severe cholangitis occurred 4 h after gastrointestinal barium X-ray examination was done. In the process of barium examination, barium refluxed to the CBD, gallbladder and intrabiliary bile duct. Barium examination may induce severe cholangitis in patients after EST. Although the incidence is low, we should avoid early barium examination in patients after EST. The patient was younger than 50 years, if choledochotomy was performed and the sphincter of Oddi was preserved, reflux cholangitis might not occur.

In conclusion, EST, as a standard treatment of choledocholithiasis, destroyed the integrity of sphincter of Oddi which may induce reflux cholangitis. As a motivation factor, barium examination may induce severe cholangitis in patients after EST, although rare, it should be performed cautiously. Cautions should also be used when EST was performed in patients younger than 50 years to avoid the damage to the sphincter of Oddi.

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Hendrik-Tobias Arkenau, MD, Sarah Cannon Research UK, 93 Harley Street, W1G 6AD London, United Kingdom

Vezali Elena, MD, Department of Hepatology, "Hygeia" Diagnostic and Therpaeutic Center of Athens, Eruthrou Staurou 4, 15123 Marousi, Greece

Dr. Pramod Kumar Garg, MD, Associate Professor, Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi 110029, India

Grigoriy E Gurvits, MD, Department of Gastroenterology, St. Vincent's Hospital and Medical Center, New York Medical College, 153 West 11th Street, Smith 2, New York, NY 10011, United States

Marek Hartleb, Professor, Department of Gastroenterology, Silesian Medical School, ul. Medyków 14, 40-752 Katowice, Poland

Jens Hoeppner, MD, Assistant Professor, Depatment for General- and Visceral Surgery, Universitaetsklinikum Freiburg, Hugstetterstr. 55, 79106 Freiburg, Germany

Jin-Hong Kim, Professor, Department of Gastroenterology, Ajou University Hospital, San 5, Wonchon-dong, Yeongtong-gu, Suwon 442-721, South Korea

Tsuneo Kitamura, Associate Professor, Department of Gastroenterology, Juntendo University Urayasu Hospital, Juntendo University School of Medicine, 2-1-1 Tomioka, Urayasu-shi, Chiba 279-0021, Japan

Teng-Yu Lee, MD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Taichung Veterans General Hospital, 160, Sec. 3, Taichung Harbor Road, Taichung 407, Taiwan, China

Yu-Yuan Li, Professor, Department of Gastroenterology, First Municipal People's Hospital of Guangzhou, 1 Panfu Road, Guangzhou 510180, Guangdong Province, China

Tamir Miloh, MD, Associate Professor, Director of Pediatric Liver/Liver Transplantation Program, Division of Gastroenterology, Phoenix Children's Hospital, 1919 E Thomas Rd, Main Building, Second Floor, Phoenix, AZ 85016, United States

Robert C Moesinger, MD, FACS, Northern Utah Surgeons, Adjunct Assistant Professor, Department of Surgery, University of Utah, 4403 Harrison Blvd. 1635, Ogden, UT 84403, United States

Nadia Peparini, MD, PhD, Department of General Surgery "Francesco Durante", La Sapienza University, Viale del Policlinico,155, 00161 Rome, Italy

Dr. Mihaela Petrova, MD, PhD, Clinic of Gastroenterology, Medical Institute, Ministry of Interior, 1606 Sofia, Bulgaria

Ji Kon Ryu, Professor, Department of Internal Medicine, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-744, South Korea

Ala Sharara, MD, FACG, AGAF, Professor of Medicine, Head, Division of Gastroenterology, American University of Beirut Medical Center, Consulting Professor, Duke University Medical Center, PO Box 11-0236, Riad El Solh 110, Beirut 72020, Lebanon

Julian Swierczynski, MD, PhD, Professor, Department of Biochemistry, Medical University of Gdansk, 80-211 Gdansk, Poland

Eduardo Garcia Vilela, Professor, PhD, Department of Internal Medicine, Faculty of Medicine, Federal University of Minas Gerais, Avenida Professor Alfredo Balena, 190/2 andar, Belo Horizonte 30130-100, Brazil

Jian Wu, Associate Professor of Medicine, Internal Medicine/Transplant Research Program, University of California, Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento, CA 95817, United States

Chun-Qing Zhang, Professor, Department of Gastroenterology, Shandong Provincial Hospital, Jinan 250021, Shandong Province, China

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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Editor-in-chief
Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University of Pisa, Director of General Medicine 2 Unit University Hospital of Pisa, Via Roma 67, 56124 Pisa, Italy

Myung-Hwan Kim, MD, PhD, Professor, Head, Department of Gastroenterology, Director, Center for Biliary Diseases, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-2dong, Songpa-gu, Seoul 138-736, South Korea

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Matt D Rutter, MBBS, MD, FRCP, Consultant Gastroenterologist, Senior Lecturer, Director, Tees Bowel Cancer Screening Centre, University Hospital of North Tees, Durham University, Stockton-on-Tees, Cleveland TS19 8PE, United Kingdom

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Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
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World Journal of Gastroenterology
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Fax: +86-10-85381893
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EDITORIAL

Enhanced recovery programme in colorectal surgery: Does one size fit all?

Alison Lyon, Christopher J Payne, Graham J MacKay

Alison Lyon, Specialty Registrar in General Surgery, West of Scotland Rotation, Glasgow G4 0SF, United Kingdom
Christopher J Payne, Graham J MacKay, Lister Department of Surgery, Glasgow Royal Infirmary, Glasgow G4 0SF, United Kingdom
Author contributions: Lyon A acted as first author of the paper; Payne CJ helped research, write and edit the paper; MacKay GJ set the framework and performed the final edit of the paper.
Correspondence to: Graham J MacKay, MBChB, FRCS, MD, Consultant Colorectal Surgeon, Honorary Senior Lecturer, Lister Department of Surgery, Glasgow Royal Infirmary, 84 Castle Street, Glasgow G4 0SF, United Kingdom. drgjmac@hotmail.com
Telephone: +44-141-2320852 Fax: +44-141-2114880
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Abstract

Enhanced recovery after surgery (ERAS) employs a multimodal perioperative care pathway with the aim of attenuating the stress response to surgery and accelerating recovery. It has been difficult to determine the relative importance of some of the individual components of these pathways such as epidural analgesia and laparoscopic colorectal surgery. Some argue that only a rigid adherence to the published ERAS protocol can achieve the proposed benefits of fast-track surgery. In this article, we explore some of the areas where the evidence base may be changing and ask whether a more flexible and individualised approach should be considered.

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Key words: Enhanced recovery; Fast-track; Laparoscopic; Intravenous fluid; Postoperative analgesia

Peer reviewer: George Sgourakis, MD, PhD, FACS, 2nd Surgical Department and Surgical Oncology Unit, Red Cross Hospital, 11 Mantzarou Str., Neo Psychiko, 15451 Athens, Greece

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INTRODUCTION

Enhanced recovery after surgery (ERAS) or "fast track surgery" is a concept first described by Kehlet[1] in the early 1990's. The approach employs a multimodal perioperative care pathway with the aim of attenuating the stress response to surgery and accelerating recovery[2]. Implementation of enhanced recovery protocols has led to improved outcomes across a range of different specialties including reductions in postoperative morbidity and hospital stay[3-5].

The fundamental premise of ERAS is the incorporation of evidence-based practice. It would seem to follow therefore that the evolution of enhanced recovery guidelines should be dynamic, allowing modifications of certain aspects of the program as new data becomes available. Some authors have advocated a rigid adherence to the ERAS protocol, citing study data that demonstrates a proportional relationship between deviation from the protocol and increased morbidity[6]. However, as evidence for components of the ERAS protocol change, it may be that a more flexible and individualised approach should be considered.

PERIOPERATIVE FLUID ADMINISTRATION

Traditionally, patients undergoing major colorectal surgery have received liberal volumes of intravenous fluids[7]. Excess intravenous fluid during and after surgery has been associated with delayed gut function and increased complication rates[8-10]. Fluid restriction has been proposed as a possible method of improving recovery and reducing postoperative complications. Brandstrup *et al*[10] found that randomising patients undergoing elective colorectal

resection to a restricted fluid protocol reduced cardiopulmonary and wound morbidity. MacKay *et al*[11] found no difference in recovery of gastrointestinal function or time to discharge with postoperative fluid restriction while using a conservative intra-operative protocol.

Goal directed fluid therapy *via* oesophageal Doppler (OD) monitoring offers an opportunity to individualise peri-operative fluid administration. OD provides a real time representation of haemodynamic function, and has been shown to be comparable with other methods for estimating cardiac output such as LIDCO. A number of studies have shown that goal-directed fluids reduce morbidity, critical care admissions,and hospital stay[12]. It is not clear however whether these benefits are still significant within an enhanced recovery protocol.

Other goal-directed techniques employ central venous oxygen saturation ($ScvO_2$) as a surrogate for mixed venous oxygen saturation. $ScvO_2$ is related to tissue oxygenation and so can be used to titrate oxygen and fluid therapy, particularly in the immediate postoperative period. This approach requires central venous access which is not always available as some groups have developed a less invasive approach to monitoring. While a number of different fluid protocols have been proposed, the optimal approach is still unclear.

EVOLVING POSTOPERATIVE ANALGESIA

Epidural analgesia was considered central to early ERAS protocols, since it reduces the endocrine-mediated stress response[13,14], and improves postoperative intestinal function[15]. Epidural analgesia also provides superior pain control to systemic opiates, particularly in the first 24-36 h after surgery[16]. Data on the effect of epidural analgesia come predominantly from studies in open surgery while the benefits in laparoscopic surgery are less clear. Levy *et al*[17,18] performed a meta-analysis to address this question but concluded that there was a paucity of quality data. The authors subsequently performed a study in which patients were randomised to receive epidural, spinal or patient-controlled opiate analgesia following elective laparoscopic colorectal resection. They demonstrated a significantly longer hospital stay, time to return of bowel function and duration of nausea in the epidural group. Intra-thecal morphine has been proposed as an alternative[19]. A meta-analysis provides encouraging results in patients undergoing abdominal surgery; reduced post-operative pain in the first 48 h and significantly reduced opiate consumption compared with systemic opiates[20]. Transversus abdominus plane blocks have also been gaining in popularity although comparative data is still lacking[21].

Epidurals can cause vasodilatation and hypotension[22], resulting in excess fluid challenges, third space shift and fluid overload. As studies emerge demonstrating benefits of alternative analgesic techniques, it does raise the question: Should epidural analgesia be the standard technique for all colorectal resections? Perhaps a more individualised approach dependent on the procedure, use of laparoscopy and placement of incisions should be considered. In this way more patients may be able to avoid potential complications while maintaining adequate analgesia and facilitating early mobilisation.

LAPAROSCOPIC AND OPEN SURGERY IN ENHANCED RECOVERY

The adoption of laparoscopic techniques within colorectal surgery came at a similar time to the introduction of "fast-track" surgery. Early studies examining the effect of laparoscopic surgery showed clear superiority in short term outcomes when compared with open surgery using traditional recovery technique[23,24]. Patients undergoing laparoscopic surgery have reduced in-patient stays, less morbidity and improved postoperative pain[25,26]. What is less clear is how much of the benefit is attributable to laparoscopy and how much is an effect of differing peri-operative care pathways.

Since these early trials there have been a number of small trials comparing laparoscopic and open colorectal surgery within an enhanced recovery setting with conflicting results[11,26-28]. Most recently, Vlug *et al*[29] performed a four-armed randomised study of patients undergoing either open or laparoscopic surgery, in an enhanced recovery or standard recovery programme. They demonstrated a significantly faster recovery time following colonic surgery in those patients undergoing laparoscopic procedures within an ERAS programme.

What is clear is that there are still a number of areas within the enhanced recovery protocol where the evidence-base continues to change. The relative contributions of different facets of the protocol also remain to be determined. While this is the case we should accept a flexible approach to facilitate the adoption of techniques supported by randomised data. There may also be scope for a degree of individualisation to reflect the wide range of patients and procedures to which enhanced recovery is now being applied.

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Role of Smad7 in inflammatory bowel diseases

Giovanni Monteleone, Roberta Caruso, Francesco Pallone

Giovanni Monteleone, Roberta Caruso, Francesco Pallone, Department of Internal Medicine, University Tor Vergata of Rome, 00133 Rome, Italy
Author contributions: Monteleone G, Caruso R and Pallone F contributed equally to draft and revise the paper.
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Correspondence to: Giovanni Monteleone, MD, PhD, Department of Internal Medicine, University Tor Vergata of Rome, 00133 Rome, Italy. gi.monteleone@med.uniroma2.it
Telephone: +39-6-72596158 Fax: +39-6-72596391
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Abstract

Crohn's disease and ulcerative colitis, the major forms of inflammatory bowel diseases (IBD) in man, are complex diseases in which genetic and environmental factors interact to promote an excessive mucosal immune response directed against normal components of the bacterial microflora. There is also evidence that the pathologic process is due to defects in counter-regulatory mechanisms, such as those involving the immunosuppressive cytokine transforming growth factor (TGF)-β1. Indeed, studies in human IBD tissues and murine models of colitis have documented a disruption of TGF-β1 signalling marked by a block in the phosphorylation of Smad3, a signalling molecule associated with the activated TGF-β receptor, due to up-regulation of Smad7, an intracellular inhibitor of Smad3 phosphorylation. Knock-down of Smad7 with a specific antisense oligonucleotide restores TGF-β1/Smad3 signalling, thus resulting in a marked suppression of inflammatory cytokine production and attenuation of murine colitis. These findings together with the demonstration that Smad7 antisense oligonucleotide is not toxic when administered in mice have paved the way for the development of a Smad7 antisense oligonucleotide-based pharmaceutical compound that is now ready to enter the clinics. In this article we review the available data supporting the pathogenic role of Smad7 in IBD and discuss whether and how Smad7 antisense therapy could help dampen the ongoing inflammation in IBD.

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Key words: Inflammatory bowel diseases; Gut inflammation; Transforming growth factor-β1; Smad7; Antisense oligonucleotides

Peer reviewers: Rasmus Goll, MD, Department of Gastroenterology, University Hospital of North Norway, Sykehusveien, 9038 Tromso, Norway; José Manuel Martín-Villa, Professor, Inmunología Unit, Facultad de Medicina, Universidad Complutense de Madrid, Pabellón V, planta 4, 28040 Madrid, Spain

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the major forms of inflammatory bowel diseases (IBD) in humans, are chronic inflammatory disorders of the gastrointestinal tract. In CD, the inflammation is typically transmural and can affect the gastrointestinal tract anywhere from the mouth to the anus. In contrast, the inflammatory lesion in UC is restricted to the mucosa and extends proximally from the rectum to involve all or part of the colon[1]. The cause of both IBD remains unknown even though there is evidence that the pathologic process results from the interaction of genetic, environmental and immunological factors[2-5]. Studies conducted in experimental models of colitis also suggest that IBD-associated tissue damage is due to an excessive immune response directed against to normal constituents of the bacterial microflora, which is inappropriately controlled

by counter-regulatory mechanisms[6,7]. An example of such mechanisms is that involving transforming growth factor (TGF)-β1, a pleiotropic cytokine with potent immune-suppressive activity. Indeed mice deficient in TGF-β1 or unable to respond to the cytokine develop intestinal inflammation[8-11], while induction of TGF-β1 protects or attenuates colitis in some murine models of IBD[12-14]. TGF-β1 activity is mediated by binding of the cytokine to a heterodimeric transmembrane serine/threonine kinases receptor, consisting of type Ⅰ (TGF-βRⅠ) and type Ⅱ (TGF-βRⅡ) subunits[15,16]. In particular, binding of TGF-β1 to TGF-βRⅡ promotes phosphorylation and activation of TGF-βRⅠ, which in turn phosphorylates/activates Smad2 and Smad3. These two Smad proteins form then a heterocomplex with Smad4, which translocates to the nucleus and regulates the functional activities of many target genes[15-17]. Smad3-deficient mice exhibit a diminished cell responsiveness to TGF-β1 and spontaneously develop a chronic inflammation in the stomach and colon, supporting the anti-inflammatory role of TGF-β1-associated Smad3 in the gastrointestinal tract[18]. In this article we review the current information on the activity of TGF-β1 in IBD and murine models of colitis and discuss data showing that restoring TGF-β1 signaling is sufficient to dampen intestinal inflammation.

DEFECTIVE TGF-β1 ACTIVITY ASSOCIATES WITH HIGH SMAD7 IN IBD

Active TGF-β1 is highly produced in the human gastrointestinal tract of healthy individuals and supposed to play a decisive role in negatively controlling inflammatory pathways[19]. Lamina propria mononuclear cells (LPMC) isolated from the normal human colon express high levels of phosphorylated (p)-Smad3, and respond to exogenous TGF-β1 with a marked inhibition of inflammatory cytokines[19]. On the other hand, neutralization of endogenous TGF-β1 in cultures of normal mucosal explants and LPMC results in enhanced synthesis of inflammatory cytokines[19]. A different scenario emerges when p-Smad3 is analyzed in LPMC and tissue samples of patients with IBD. Indeed, in both CD and UC, phosphorylation of Smad3 is markedly reduced as compared to normal controls, despite TGF-β1 is produced at high level in these patients[20,21]. Moreover, IBD LPMC do not phosphorylate Smad3 and produce huge amounts of inflammatory cytokines when stimulated with exogenous TGF-β1[20-22].

The Smad family also includes two Smad inhibitors, namely Smad6 and Smad7. Smad7 associates stably with the ligand-activated TGF-β1R I complex and blocks phosphorylation of Smad2/Smad3[15,23]. Attenuation of TGF-β1 signaling by Smad7 is also dependent on the ability of Smad7 to induce degradation of TGF-β1RI after recruitment of E3 ubiquitin ligases and to recruit protein phosphatase 1 to the receptor complex *via* interaction with growth arrest and DNA damage protein 34[24]. Since up-regulation of Smad7 associates with inhibition of TGF-β1-induced Smad2/3 activation, we explored the possibility that the diminished activation of TGF-β1-associated Smad signaling in IBD could be related to high Smad7. To this end, we examined Smad7 in the same intestinal samples analyzed for Smad3 phoshorylation. Smad7 was over-expressed in whole mucosal and LPMC samples of CD patients and UC patients as compared to controls[20]. Interestingly, silencing of Smad7 with an anti-sense oligonucleotide restored the responsiveness of IBD LPMC to exogenous TGF-β1, as indicated by the enhanced phosphorylation of Smad3 and diminished synthesis of inflammatory cytokines[20]. Similar results were seen in *ex-vivo* organ cultures of CD mucosal explants, in which inhibition of Smad7 with antisense oligonucleotide associated with high Smad3 phosphorylation and decreased inflammatory cytokine production. These effects were blocked by a neutralizing TGF-β1 antibody, clearly indicating that Smad7 knockdown allows endogenous TGF-β1 to act and suppress inflammatory signals[20].

High Smad7 expression and defective TGFβ-associated Smad3 activation are not however specific hallmarks of IBD, because similar alterations were documented in the stomach of patients with *Helicobacter pylori*-related gastritis[25]. By contrast, Smad7 expression is not up-regulated in the duodenum of patients with active celiac disease[26], raising the possibility that induction of Smad7 in the gut is not an epiphenomenon of the ongoing inflammation but rather can reflect changes in the local inflammatory milieu.

SMAD7 EXPRESSION IS POST-TRANSCRIPTIONALLY REGULATED IN IBD

Further experimentation was performed to understand how Smad7 expression is regulated in IBD. Since experiments with cell lines have shown that Smad7 can be induced by activators of nuclear factor-κB [e.g., tumor necrosis factor (TNF)-α and interleukin (IL)-1β] and STAT-1 [e.g., interferon (IFN)-γ and IL-7] and these transcription factors are hyper-activated in IBD[20,27], we initially hypothesized that Smad7 in CD and UC tissue was regulated by these signaling pathways. However, functional studies showed that Smad7 protein expression remained unchanged in IBD LPMC following treatment with IFN-γ/Stat1 or TNF-α/NF-kB inhibitors[20,27]. Smad7 is also rapidly induced by TGF-β1-driven Smad3 signaling, thus representing an important effector in the feedback loop that restrains TGF-β1 activity[23]. We feel however it is unlikely that high Smad7 is induced by TGF-β1 in IBD, because p-Smad3 was reduced in samples exhibiting enhanced Smad7 expression[20]. Analysis of Smad7 RNA expression revealed that IBD and control samples contained similar levels of transcripts, raising the possibility that, in IBD, Smad7 is post-transcriptionally regulated[28]. Indeed, Smad7 was found to be ubiquitinated and targeted for proteasome

degradation in control but not IBD samples. Difference in the level of ubiquitination of Smad7 between IBD and controls seems to be secondary to the different status of acetylation of the protein, because acetylation and ubiquitination compete for the same lysine residues of Smad7[29,30]. Thus, acetylation prevents ubiquitination and protects Smad7 protein against proteasomal degradation[27]. Smad7 was highly acetylated *in vivo* in IBD but not control samples[28]. We have also shown that in IBD, the transcriptional coactivator p300 interacted with and promoted Smad7 acetylation, and that silencing of p300 diminished acetylation and expression of Smad7[28].

INHIBITION OF SMAD7 ATTENUATES GUT INFLAMMATION IN MICE

The mouse models of Th1 or Th2-mediated colitis induced by intra-rectal administration of trinitrobenezene sulfonic acid (TNBS) or oxazolone and showing immunological similarities with CD or UC, respectively[13,31,32], were used to confirm the inflammatory role of Smad7. In both these models, colitis was marked by enhanced production of TGF-β1, reduced phosphorylation of Smad3 and high expression of Smad7[33]. Oral Smad7 antisense oligonucleotide was taken-up by epithelial cells and LPMC in the small intestine and colon and induced no toxicity. This treatment reduced Smad7 and restored TGF-β1-associated p-Smad3 expression and ameliorated both forms of colitis[33]. Analysis of inflammatory cytokines in the colon of treated mice revealed that restoration of TGF-β1 signaling by inhibition of Smad7 resulted in a significant down-regulation of IL-12 and IFN-γ and reduced expression of Th1-associated transcription factors (i.e., T-bet and Stat1) in TNBS-colitis, and reduced production of IL-4 in mice with oxazolone-induced colitis[33]. These studies suggest that resolution of gut inflammation may be accomplished by down-regulating Smad7 and allowing endogenous TGF-β1 to inhibit inflammatory pathways which promote tissue injury.

SMAD7 ANTISENSE IS SAFE AND TOLERATED IN PATIENTS WITH CD

Altogether the above data indicate that Smad7 is a molecular target for direct therapeutic interventions in IBD. To this end, we have recently developed a pharmaceutical compound containing the Smad7 antisense oligonucleotide and formulated it as a solid oral dosage form. The formulation is protected by an external coating, which allows the antisense oligonucleotide to transit through the stomach and proximal small intestine and reach the terminal ileum and right colon where the active compound is released[34]. To examine whether this drug was safe and tolerated, we conducted a phase 1, open-label study in patients with active, steroid-dependent/resistant CD. Fifteen patients were allocated to three treatment groups receiving oral Smad7 antisense oligonucleotide, once daily for 7 d at doses of 40, 80 or 160 mg. The drug was well-tolerated and no patient experienced serious adverse event (AE). Twenty-five AEs were registered in 11 patients, but the majority of AEs was mild and considered unrelated to treatment. Interestingly, treatment associated with a significant reduction in the fraction of circulating CCR9-positive T cells secreting IFN-γ and IL-17A[34]. This finding could be therapeutically relevant as CCR9-positive T cells represent a subset of inflammatory T cells with gut homing properties, which are increased in the active phases of CD[35]. Moreover, administration of Smad7 antisense oligonucleotide to patients resulted in a significant clinical benefit[34].

CONCLUSION

TGF-β1 plays a critical role in the control of gut immune homeostasis and therefore defects in the production and/or activity of the cytokine inevitably associate with pathological responses[8,9,36]. This is for example seen in the inflamed gut of IBD patients and mice with experimental colitis, where TGF-β1 is unable to signal and dampen inflammatory signals due to high Smad7, a protein which binds to TGF-βR and prevents TGF-β1/Smad3 signaling[20,21,33]. Such an alteration is not however irreversible, as knockdown of Smad7 with a specific antisense oligonucleotide restores TGF-β1 activity, inhibits inflammatory cytokine production and attenuates colitis in mice[20,33]. In line with this, results of an exploratory phase 1 trial, testing the Smad7 antisense oligonucleotide in active CD patients, have recently shown that the compound is safe and tolerable and that such treatment attenuates signs and symptoms of CD, supporting the pathogenic role of Smad7 in CD[34]. Appropriately designed placebo-controlled clinical trials will however be necessary to confirm the safety profile of this approach, show if Smad7 is a valid therapeutic target in CD, and examine the long-term risks of Smad7 knockdown. In particular, it would be relevant to exclude the possibility that restoring TGF-β1 activity favors the development of strictures[37], given that TGF-β1 is a powerful inducer of fibrogenesis in many organs[38-40]. TGF-β1 can also exert various effects on the cellular and molecular mechanisms involved in tumor development. In particular TGF-β1 can act as a tumor suppressor in early stages of tumorigenesis and promote advanced tumor cell invasiveness and metastasis[41-43]. Similarly, Smad7 may regulate differently tumorigenesis depending on the cell context analyzed. For example, over-expression of Smad7 promotes initiation of pancreatic cancer, skin cancer, scleroderma and lung cancer, associates with a worse prognosis in sporadic colorectal cancer (CRC)[44] and enhances liver metastasis in a murine model of CRC[45]. Moreover, genome-wide association studies have shown that common alleles of Smad7 can influence the risk of CRC[46] and that patients carrying on the rs12953717 single nucleotide polymorphism in the *SMAD7* gene

have increased risk of CRC. In contrast, Smad7 inhibits metastasis of human melanoma cells to bone and metastasis of human breast cancers to the lung or liver[47-50]. We have recently shown that over-expression of Smad7 in T cells and natural killer cells makes worse the course of experimental colitis yet protects from the development of colitis-associated colon-cancer[51]. Therefore, further experimentation will be also needed to ascertain if inhibition of Smad7 in the inflamed gut can enhance the risk of colitis-associated CRC.

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Levofloxacin/amoxicillin-based schemes *vs* quadruple therapy for *Helicobacter pylori* eradication in second-line

Simona Di Caro, Lucia Fini, Yayha Daoud, Fabio Grizzi, Antonio Gasbarrini, Antonino De Lorenzo, Laura Di Renzo, Sara McCartney, Stuart Bloom

Simona Di Caro, Sara McCartney, Stuart Bloom, Department of Gastroenterology, University College Hospital, London NW1 2PG, United Kingdom
Simona Di Caro, Antonino De Lorenzo, Laura Di Renzo, Division of Human Nutrition, University of Tor Vergata, 00173 Rome, Italy
Lucia Fini, Fabio Grizzi, Department of Gastroenterology and Laboratory of Molecular Gastroenterology, Humanitas Clinical and Research Center, Rozzano, 20089 Milan, Italy
Yayha Daoud, Antonino De Lorenzo, Baylor Health Care System, Institute for Health Care Research and Improvement, Dallas, TX 75246, United States
Antonio Gasbarrini, Department of Internal Medicine, Gemelli Hospital, Catholic University of Rome, 00168 Rome, Italy
Author contributions: Di Caro S and Fini L conceived and analyzed the data and wrote the paper; Daoud Y analyzed the data and performed statistical analyses; Grizzi F, Gasbarrini A, De Lorenzo A, Di Renzo L, McCartney S and Bloom S critically revised and edited the paper.
Correspondence to: Simona Di Caro, MD, Department of Gastroenterology, University College Hospital, 250 Euston Road, London NW12PG, United Kingdom. simonadicaro@yahoo.it
Telephone: +44-20-73809126 Fax: +44-20-73809217
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Abstract

Worldwide prevalence of *Helicobacter pylori* (*H. pylori*) infection is approximately 50%, with the highest being in developing countries. We compared cure rates and tolerability (SE) of second-line anti-*H. pylori* levofloxacin/amoxicillin (LA)-based triple regimens *vs* standard quadruple therapy (QT). An English language literature search was performed up to October 2010. A meta-analysis was performed including randomized clinical trials comparing 7- or 10-d LA with 7-d QT. In total, 10 articles and four abstracts were identified. Overall eradication rate in LA was 76.5% (95% CI: 64.4%-97.6%). When only 7-d regimens were included, cure rate was 70.6% (95% CI: 40.2%-99.1%), whereas for 10-d combinations, cure rate was significantly higher (88.7%; 95% CI: 56.1%-109.9%; $P < 0.05$). Main eradication rate for QT was 67.4% (95% CI: 49.7%-67.9%). The 7-d LA and QT showed comparable efficacy [odds ratio (OR): 1.09; 95% CI: 0.63-1.87], whereas the 10-d LA regimen was significantly more effective than QT (OR: 5.05; 95% CI: 2.74-9.31; $P < 0.001$; $I^2 = 75\%$). No differences were reported in QT eradication rates among Asian and European studies, whereas LA regimens were more effective in European populations (78.3% *vs* 67.7%; $P = 0.05$). Incidence of SE was lower in LA therapy than QT (OR: 0.39; 95% CI: 0.18-0.85; $P = 0.02$). A higher rate of side effects was reported in Asian patients who received QT. Our findings support the use of 10-d LA as a simple second-line treatment for *H. pylori* eradication with an excellent eradication rate and tolerability. The optimal second-line alternative scheme might differ among countries depending on quinolone resistance.

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Key words: *Helicobacter pylori*; Second-line treatment; Levofloxacin; Quadruple regimen

Peer reviewers: Dr. Shahab Abid, Associate Professor, Department of Medicine, Aga Khan University, Stadium Road, PO Box 3500, Karachi 74800, Pakistan; Siegfried Wagner, Professor, Medizinische Klinik Ⅱ, Klinikum Deggendorf, Perlasberger Str. 41, 94469 Deggendorf, Germany

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INTRODUCTION

Since 1982, when Warren and Marshall first discovered *Helicobacter pylori* (*H. pylori*) in the stomach, eradication of the infection has being recognized as crucial for prevention and treatment of gastroduodenal and more recently even extraintestinal diseases[1-4]. The worldwide prevalence of *H. pylori* infection is approximately 50%, with the highest being in developing countries[5]. Standard first-line therapy consists of a proton pump inhibitor (PPI) plus clarithromycin and amoxicillin or metronidazole[6] and achieves successful eradication in up to 80% of patients. During the past decade, an alarming decrease in eradication rates has been observed[7], mostly due to antibiotic resistance to clarithromycin and metronidazole[3,8]. Conversely, resistance to amoxicillin and tetracycline remains a rare occurrence worldwide[9-11]. Choice of a second or even third course of therapy is more controversial[6,9,12-14]. Recently, the concept of cumulative eradication rate has been introduced in treatment of *H. pylori* infection focusing on final cure rate after one or more courses of treatment. In this perspective, if the overall eradication rate is considered even after four consecutive empirical treatments, 99.5% of patients can be cured[15]. Currently, the recommended second-line regimen is a 7-14-d complex scheme consisting of PPI, bismuth, tetracycline and metronidazole[16,17]. However, bismuth salts, applied to decrease bacterial load, are no longer available in many countries[18]. Eradication rate of the quadruple regimen ranges from 65% to 80% and a high incidence of side effects are noted (up to 50% of patients), with consequently poor compliance[19-22]. For this reason, a triple therapy consisting of PPI, metronidazole and amoxicillin or tetracycline for a minimum of 7 d has been recommended as an alternative[6]. Meanwhile, new drugs, such as levofloxacin, rifabutin, furazolidone and azithromycin have been tested in various combinations and doses to overcome falling eradication rates[13,14,23-31].

Among those, levofloxacin-based second-line schemes represent the most promising alternative[32,33]. In particular, association with amoxicillin has been extensively studied, due to rare *H. pylori* resistance. Currently, a 10-d triple levofloxacin (250 mg BD)/amoxicillin (LA)-based therapy has been recommended as second-line therapy in Italy, and as third-line empirical treatment by European guidelines[6,16]. However, heterogeneity in terms of dosage, combination and duration of treatment do not allow a definitive conclusion.

Moreover, in consideration of differences in quinolone resistance among countries, the ideal second-line treatment for *H. pylori* infection may differ between areas, countries and races[10,20,34].

Here we compare eradication rates and tolerability exclusively of second-line anti-*H. pylori* LA-based triple schemes and standard quadruple therapy (QT) in European and Asian studies, in order to guide clinical decision making.

Studies providing information on use of anti-*H. pylori* LA-based triple therapy and standard quadruple scheme in second-line were identified through a systematic search in the MEDLINE and EMBASE databases by using various combinations of the terms "*H. pylori*", "levofloxacin", "amoxicillin", "quadruple", "bismuth", "second-line" and "rescue". Additionally, references of retrieved articles were screened for further studies (cross-referencing). We also performed a full manual search of all review articles, recently published editorials and all retrieved original studies presented at Digestive Disease Week, United European Gastroenterology Week, and European Helicobacter Study Group conferences. In addition, reference lists from relevant identified papers were manually searched. All original research articles and abstracts published until the 31 October 2010 were included. The search was limited to randomized controlled trials (RCTs) and studies comparing the two regimens (LA *vs* QT). Two investigators (Di Caro S and Fini L) independently extracted data by using a structured form. Only data from patients undergoing second-line eradication treatments were included in the analysis. There was a > 95% agreement in data extraction between the two investigators.

The following data were extracted from the articles: first author, year of publication, characteristics of study population (sample size, race, sex, age range and mean of study participants), therapy scheme (drugs, duration of treatment, dosage), intention-to-treat (ITT) and per-protocol eradication rates, incidence of side effects, discontinuation due to side effects.

Study outcomes for the meta-analysis were *H. pylori* eradication and incidence of adverse effects. Sub-analyses were performed comparing 7-d *vs* 10-d levofloxacin-based regimens in terms of efficacy and adverse events between European and Asian populations. Eradication rate analysis was based on ITT data. In the tolerability analysis, patients who discontinued treatment due to severe side effects were included.

The meta-analysis was performed using the Preferred Reporting Items for Systematic reviews and Meta-Analyses 2009 guidelines. Odds ratio (OR) was used as a measure of association, and summary ORs along with 95% CI were calculated based on a random-effects model using the DerSimonian and Laird methods. Heterogeneity was evaluated to establish if any clinical, methodological, or statistical variability existed among studies included. If significant heterogeneity existed, the random-effects model was used. Also, inconsistency statistic (I^2) was calculated to evaluate level of heterogeneity (0%-30%: homogeneity; 30%-50%: moderate heterogeneity; 50%-80%: substantial heterogeneity; > 80%: considerable heterogeneity). A pooled analysis was used to assess differences between levofloxacin regimens (7 d *vs* 10 d) and study populations. All statistical tests were two-sided and conducted at a significance level of 0.05. Independent academic biostatisticians from Baylor University Medical Center (Daoud Y) performed the statistical analysis. Data were analyzed with Review Manager (RevMan) 5.1 developed by the Cochrane collaboration.

Table 1 Demographic data of patients, extracted from the published papers (n = 9)

Ref.	Pts	Days of tp	Test confirming infection	Test confirming eradication	Age (yr)		Male (%)		Smokers (%)		Clinic (%)	
					L + A	Q	L + A	Q	L + A	Q	L + A	Q
Nista *et al*[21]	140	10	UBT and/or biopsy	UBT	47 ± 10	48 ± 10	47	49	NA	NA	UD = 37; DD = 33; RD = 30	UD = 40; DD = 34; RD = 26
Perri *et al*[29]	113	7	UBT	UBT	46 ± 16	45 ± 15	48	47	17	20	N = 63; OE = 3; G = 9; GU = 3; D = 10; DU = 9; O = 3	N = 67; OE = 5; G = 2; GU = 0; D = 19; DU = 5; O = 2
Bilardi *et al*[39]	90 (40)	10	UBT and/or biopsy	UBT	56 ± 10	54 ± 13	30	35	64	52	NA	NA
Gisbert *et al*[28]	500 (150)	7-10	UBT	UBT	48 ± 13[1]		39[1]		23[1]			FD = 76; DU = 24[1]
Lee *et al*[80]	126 (23)	7	Rapid urea test	UBT	50 ± 14[1]		63[1]		24[1]			GU = 44; DU = 32; GA = 11; A = 3; F = 1; FD = 9
Wong *et al*[19]	106 (61)	7	Rapid urea test and/or biopsy	UBT	47 ± 12[1]	45 ± 13[1]	44[1]	42[1]	20[1]	19[1]	N = 64; E = 6; GU = 6; DU = 24	N = 77; E = 8; GU = 0; DU = 15
Zhang *et al*[37]	95	7			49		NA	NA	NA	NA	GU/DU = 24; G = 62; GA = 14	
Jung *et al*[42]	76	7	Rapid urea test and/or UBT	Rapid urea test or UBT	48 ± 12	50 ± 10	58	58	NA	NA	GU = 39; DU = 32; GA = 10; G = 19	GU = 38; DU = 33; GA = 18; G = 11
Kuo *et al*[41]	166	7	Rapid urea test and/or biopsy or culture	Rapid urea test and/or biopsy or UBT	50 ± 12	49 ± 14	53	48	14	12	GU = 23; DU = 41; G = 36	GU = 25; DU = 40; G = 35

[1]Demographic data were reported together for both arms. In studies including patients after failure of more than one therapy, extrapolated data on patients pre-treated in second-line are reported in parenthesis; in those cases demographic data are referred to the entire study population. Pts: Patients (total number); L + A: Levofloxacin/amoxicillin-based therapy; Days of therapy (tp): Duration of L + A scheme; UBT: Urea breath test; Q: Quadruple standard therapy; UD: Ulcer-like dyspepsia; DD: Dysmotility-like dyspepsia; RD: Reflux-like dyspepsia; N: Normal; OE: Esophagitis; G: Gastritis; GU: Gastric ulcer; D: Duodenitis; O: Other; FD: Functional dyspepsia; DU: Duodenal ulcer; A: Adenoma; E: Erosion; GA: Gastric atrophy; F: Family history; NA: Not available.

TEN-DAY LEVOFLOXACIN SECOND-LINE SCHEMES DISPLAY SIGNIFICANTLY HIGHER ERADICATION RATE AND LOWER INCIDENCE OF SIDE EFFECTS

Out of 79 titles initially generated by literature searches, 14 studies (10 articles and four abstracts) fulfilling the inclusion criteria were eligible for our analysis. All studies were published from September 2003 to May 2009 and included 677 patients in the LA-based triple therapy group and 608 subjects in the QT group. First-line therapy was a standard triple anti-*H. pylori* scheme. Tolerability data were available in seven articles. Demographic and clinical data of patients available are reported in Table 1. Studies were divided into two categories based on ethnicity of the study population (Asian and Caucasian population). In all studies, *H. pylori* infection was determined by ^{13}C urea breath test (UBT), rapid urea test, histology or culture before treatment, and histology and/or ^{13}C-UBT after administration of eradication therapy. Heterogeneity of data available on levofloxacin dose, ranging from 300 mg/d to 1 g/d (Table 1), does not allow a definitive conclusion.

Data on comparison of second-line levofloxacin-based triple regimens to bismuth-based QT after failure of one cycle of eradication therapy are listed in Table 2.

In our analysis, we extrapolated exclusively the data matching our strict inclusion criteria. We also re-calculated all the eradication percentages from the included studies. In all cases, the results matched the reported data, except one.

In an Italian population study, 10-d levofloxacin-based triple scheme in association with either amoxicillin or tinidazole, was superior to both 7-d and 14-d QT. Specifically, triple therapy eradication rates were 94% with amoxicillin *vs* 63% and 68% at 7 d and 14 d, respectively for QT. A high incidence of adverse events

Table 2 Levofloxacin-based second-line therapies *vs* quadruple bismuth-based regimens for *Helicobacter pylori* treatment

Ref.	Ethnicity	Scheme	Success					Side effects			Discontinuing therapy because of side effects		
			Yes	Total	Completing	ITT %	PP %	Yes	Total	%	Yes	Total	%
Nista *et al*[21]	Caucasian	LAR (10 d; L: 500 mg OD)	66	70	70	94.3	94.3	7	70	10.0	0	70	0.0
		Q07	44	70	64	62.9	68.8	15	70	21.4	5	70	7
Perri *et al*[29]	Caucasian	LAP (7 d; L: 500 mg OD)	37	58	56	63.8	66.1	3	58	5.2	1	58	1.7
		Q07	46	55	51	83.6	90.2	17	55	30.9	3	55	5.5
Orsi *et al*[1][38]	Caucasian	LAR (12 d; L: 500 mg OD)	43	50	47	86.0	89	4	47	8.5	NA	NA	NA
		Q07	44	50	44	88.0	91	11	44	25	NA	NA	NA
Bilardi *et al*[39]	Caucasian	LAP (10 d; L: 250 mg BD)	16	23	NA	69.6	NA	-	-	-	1	44	
		Q07	6	17	NA	35.3	NA	-	-	-	1	46	
Nista *et al*[1][35]	Caucasian	LAE (10 d; L: 500 mg OD)	26	30	30	86.7	86.7	-	-	-			
		Q07	25	35	31	71.4	80.6	-	-	-			
Nista *et al*[1][36]	Caucasian	LAR (10 d; L: 500 mg OD)	42	46	46	91.3	91.3	-	-	-			
		LAR (7 d; L: 500 mg OD)	37	50	50	74.0	74.0	-	-	-			
		Q07	34	50	46	68	73.9	-	-	-			
Gisbert *et al*[28]	Caucasian	LAO (7 or 10 d; L: 500 mg BD)	83	112	NA	74	NA	39	112	34.8	NA	NA	NA
		Q07	21	38	NA	55	NA	6	38	15.8	NA	NA	NA
Wong *et al*[1][44]	Asian	LALa (7 d; L: 500 mg BD)	21	33	NA	63.6	NA	-	-	-			
		Q07	22	30	NA	73.3	NA	-	-	-			
Lee *et al*[80]	Asian	LAR (10 d; L: 200 mg BD)	5	9	8	55.6	62.5	-	-	-			
		Q07	4	14	10	28.6	40.0	-	-	-			
Wong *et al*[19]	Asian	LALa (7 d; L: 500 mg BD)	19	31	NA	61.3	NA	18	54[2]	33.3	NA	NA	NA
		Q07	26	30	NA	86.7	NA	21	52[2]	40.4	NA	NA	NA
Zhang *et al*[43]	Asian	LAE (7 d; L: 500 mg OD)	42	49	NA	85.7	NA						
		Q07	30	44	NA	68.2	NA						
Zhang *et al*[37]	Asian	LAE (7 d; L: 500 mg OD)	42	48	46	87.5	91.3	7	48				
		Q07	33	47	34	70.2	97.0	15	47				
Jung *et al*[42]	Asian	LAP (7 d; L: 300 mg BD)	16	31	30	51.6	53.3	3	30	10.0	0	31	0.0
		Q07	22	45	35	48.9	62.9	11	35	31.4	1	45	2.2
Kuo *et al*[41]	Asian	LAE (7 d; L: 500 mg OD)	58	83	77	69.9	75.3	10	80	12.5	1	83	
		Q07	53	83	63	63.9	84.1	25	71	35.2	5	83	

[1]Data arisen from abstract publication; [2]side effects calculated on the entire study population (not stratified for number of failed previous therapy). L: Levofloxacin; OD: Once daily; BD: Twice daily; LA/La: Levofloxacin/Lansoprazole; ITT: Intention-to-treat eradication rates; PP: Per-protocol; NA: Not available; LAR: Levofloxacin/amoxicillin/rabeprazole; LAO: Levofloxacin/amoxicillin/omeprazole; LAE: Levofloxacin/amoxicillin/esomeprazole; LAP: Levofloxacin/amoxicillin/pantoprazole.

(33%) was observed in the 14-d QT group, although the same scheme administered for 7 d was well tolerated. Dropouts and severe adverse events were observed exclusively in the quadruple regimen[21]. The same authors compared two 10-d levofloxacin-based (500 mg OD) triple schemes in combination with either amoxicillin or azithromycin with the standard QT regimen. Eradication rates of levofloxacin-based groups were higher, even if not significantly, compared with standard QT (86.6% and 71.4%, respectively), but incidence of side effects was significantly lower (26.6% *vs* 60%). Dropouts occurred only in the QT group[35,36].

In terms of duration of treatment, the same researchers compared efficacy and tolerability of 7 d and 10 d levofloxacin/amoxicillin-based treatment to 7 d QT, demonstrating a higher cure rate for the triple 10-d levofloxacin regimen (91.3%) compared to both the 7-d levofloxacin-based or QT regimen (74% and 68%, respectively), with optimal tolerability[35,36]. Zhang *et al*[37] also confirmed similar results.

Orsi *et al*[38] have studied efficacy of a 12-d treatment course by comparing the levofloxacin/amoxicillin-based regimen with QT. Both regimens were equally effective (86% *vs* 88%) but the triple regimen was better tolerated (incidence of side effects: 8.6% *vs* 24%).

A drastic difference in eradication rates were observed in another Italian study in which the levofloxacin-based triple and quadruple regimens achieved an eradication rate of 70% *vs* 35%, respectively, in second-line, even if patients were given a full-dose course of PPI for 1 wk prior to bismuth therapy. Interestingly, levofloxacin-based triple regimen was successful in most patients with both metronidazole- and clarithromycin-resistant strains, whereas QT was less effective, particularly when metronidazole resistance occurred[39].

Bilardi *et al*[39] used LA-based triple therapy both in first- and second-line compared to standard first-line triple and quadruple second-line regimens, respectively. In first-line, eradication rate of levofloxacin-based and standard triple regimen was 69.8% and 74%, respectively, while as rescue regimen, levofloxacin-based therapy achieved an eradication rate of 62.5% compared to 40% for the standard QT regimen.

Gisbert e*t al*[40] compared 7-d levofloxacin-based therapy with a quadruple ranitidine bismuth citrate-based regimen and reported an identical eradication rate of

68% for both regimens, with similar incidence of side effects (38% *vs* 36%, respectively). Nevertheless, the antisecretory activity of ranitidine might have contributed to the eradication rate in the QT regimen. Conversely, dose of levofloxacin (500 mg BD) and duration (7 d) of treatment might have affected efficacy (shorter treatment) and tolerance (high dosage) in the triple regimen[40].

In the study by Kuo *et al*[41], levofloxacin triple regimen and QT eradication rates were 69.9% *vs* 63.9%, respectively, and although compliance was similar, incidence of side effects was 12.6% in the LA *vs* 35.2% in the QT groups. In this study, levofloxacin resistance was analyzed and was a crucial predictor for eradication failure (21.2% of patients).

In a Korean trial, 7 d levofloxacin-based therapy and standard QT achieved comparable but low cure rates (51.6% *vs* 48.9%), even if the triple regimen was better tolerated (adverse events incidence: 10% *vs* 31.4%)[42]. Another Asian trial confirmed the high cure rate of levofloxacin-based therapy for 7 d (85.7%)[43].

Finally, few studies have demonstrated superiority of the recommended QT regimen compared with the levofloxacin triple regimen. QT was superior to levofloxacin-based regimen for persistent *H. pylori* infection in the study conducted by Perri *et al*[29] (eradication rate: 63% in the levofloxacin group). In this trial bismuth- and ranitidine/bismuth citrate-based QT regimens achieved a cure rate of 83% and 85%, respectively, although a higher incidence of side effects was observed in the bismuth-treated group (30.9% *vs* 5.1%). Conversely, adverse events occurred in 3.3% of patients treated with levofloxacin.

Wong *et al*[44] reported an eradication rate of 73% and 64% in second-line for QT and levofloxacin-based regimens, respectively. Similarly, in a study conducted in Hong Kong, second-line 7-d triple levofloxacin-based therapy achieved an eradication rate of 61% *vs* 87% with QT, due to resistance to levofloxacin (18% of patients)[19]. However, the study included a small sample of refractory patients and eradication rate was superior in subjects in the LA group with dual resistance to metronidazole and clarithromycin respect to antibiotics resistant patients in Q group (79% *vs* 65%, respectively). However, treatment choice was based on antibiotic susceptibility testing and might not reflect common clinical practice[19].

Results of our meta-analysis are summarized in Figure 1. Overall eradication rate (pooled data) with levofloxacin was 76.5% (95% CI: 54.4%-97.6%). When exclusively 7-d regimens were included, cure rate was 70.6% (95% CI: 40.2%-99.1%), whereas for the 10-d regimen, eradication rate was significantly higher (88.7%; 95% CI: 56.1%-109.9%; $P < 0.05$). Mean eradication rate for QT was 67.4% (95% CI: 42.4%-91.6%).

The meta-analysis showed a trend indicating superiority of any LA regimens (7, 10 or 12 d) to QT (OR: 1.59; 95% CI: 0.98-2.58; $P = 0.06$; Figure 1A). Both 7-d LA and QT regimens showed comparable efficacy (OR: 1.09; 95% CI: 0.63-1.87; $P = 0.40$; Figure 1B), whereas the 10-d LA regimen was significantly more effective (OR: 5.05; 95% CI: 2.74-9.31; $P < 0.00\,001$; Figure 1C).

No differences were reported in QT eradication rates between Asian and European studies, whereas LA regimens were more effective in Caucasians (78.3% *vs* 67.7%; $P = 0.05$). Of note, if data on comparison between LA and QT regimens were stratified by ethnicity (Figure 1A), any levofloxacin regimen was more effective in the Caucasian population ($P = 0.09$). Those findings were not confirmed when only 7-d levofloxacin regimens were included (Figure 1B). No data were available on 10-d levofloxacin regimens in Asian populations (Figure 1C).

Mean incidence of side effects (pooled data) was lower in LA regimens (13.7%, 95% CI: 12%-24%) compared with standard QT (27.2%, 95% CI: 24%-34%). The OR for this comparison was 0.39 (95% CI: 0.18-0.85; $P = 0.02$) at meta-analysis (Figure 2). When incidence of side effects (pooled data) was stratified by ethnicity, no differences were registered between the two geographical areas for the levofloxacin regimens (14.8% *vs* 12.1%, respectively in Europe and Asia), whereas a slightly higher rate was reported in the Asian population treated with QT (32.5% *vs* 23.3%, $P = 0.09$). Moreover, the lower risk of occurrence of side effects in the levofloxacin arms overall was mainly due to the Asian studies contribution.

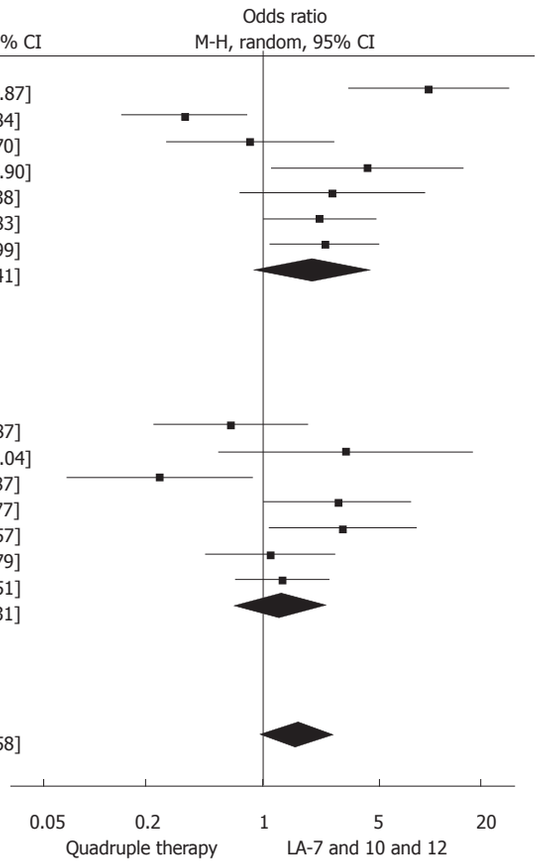
DISCUSSION

Antibiotic resistance is the main cause of failure in curing *H. pylori* infection[45-49]. Other factors determining eradication rate are: *H. pylori* strain, patient compliance, and properties of drugs administered[2].

Levofloxacin, a second-generation fluoroquinolone, with a broad spectrum of activity against Gram-positive and Gram-negative bacteria[50-52], is a recognized antimicrobial alternative to the standard antibiotics used to treat *H. pylori* infection. Levofloxacin is mainly indicated to treat infections of respiratory and genitourinary tract, skin and skin structures[33,51,53-55]. In recent years, its role has been successfully extended to treatment of *H. pylori* infection and has been included as treatment of choice in guidelines[15,33,56]. Its optimal tolerance spectrum, with the most frequent adverse events being nausea and diarrhea, makes it a safe alternative[57]. Only a few cases of QTc prolongation, seizures, glucose disturbances and tendonitis have been reported[33,51,56]. Although primary resistance is infrequent, resistance to quinolones is easily acquired in areas of high consumption[58]. In Asian countries, levofloxacin resistance has recently increased[59,60]. In particular, in Hong Kong, Korea and Japan, estimated *H. pylori* resistance rates for levofloxacin are 11.5%, 21.5% and 15% respectively[61,62]. In the Chinese adolescent population, all *H. pylori* strains were found to be resistant to levofloxacin[63,64]. In Europe, such as Spain, France, Netherlands, Austria and Portugal, fluoroquinolone resistance is less than 10%[65-69]. However, despite a reported levofloxacin resistance rate of 18%, Italian guidelines recommend levofloxacin regimens for second-line therapy, with optimal results[16]. Moreover, antibiotic

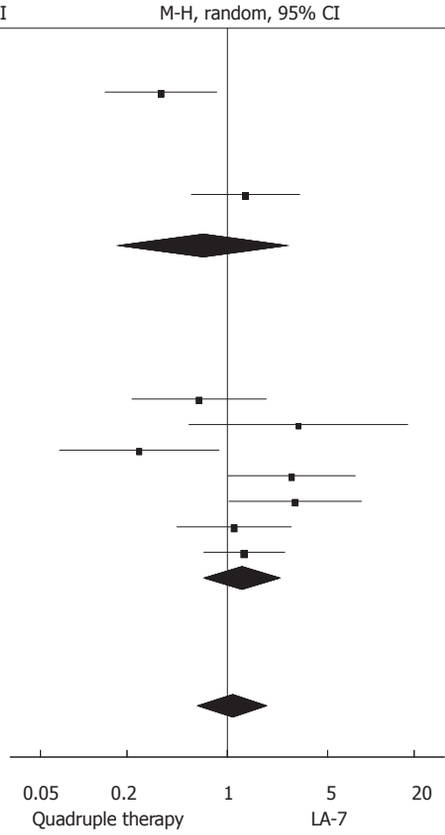
A

Study or subgroup	LA 7 and 10 and 12 Events	Total	Quadruple therapy Events	Total	Weight	Odds ratio M-H, random, 95% CI
1.4.1 Caucasian						
Nista *et al*[21]	66	70	44	70	6.9%	9.75 [3.18, 29.87]
Perri *et al*[29]	37	58	46	55	8.0%	0.34 [0.14, 0.84]
Orsi *et al*[38]	43	50	44	50	6.7%	0.84 [0.26, 2.70]
Bilardi *et al*[39]	16	23	6	17	6.0%	4.19 [1.10, 15.90]
Nista *et al*[35]	26	30	25	35	6.2%	2.60 [0.72, 9.38]
Nista *et al*[36]	79	96	34	50	8.4%	2.19 [0.99, 4.83]
Gisbert *et al*[28]	83	112	21	38	8.6%	2.32 [1.08, 4.99]
Subtotal (95% CI)		439		315	50.7%	1.99 [0.90, 4.41]
Total events	350		220			
Heterogeneity: Tau^2 = 0.87; χ^2 = 26.07, df = 6 (P = 0.0002); I^2 = 77%						
Test for overall effect: Z = 1.69 (P = 0.09)						
1.4.2 Asian						
Wong *et al*[44]	21	33	22	30	7.1%	0.64 [0.22, 1.87]
Lee *et al*[80]	5	9	4	14	4.5%	3.13 [0.54, 18.04]
Wong *et al*[19]	19	31	26	30	6.2%	0.24 [0.07, 0.87]
Zhang *et al*[43]	42	49	30	44	7.3%	2.80 [1.01, 7.77]
Zhang *et al*[37]	42	48	33	47	7.2%	2.97 [1.03, 8.57]
Jung *et al*[42]	16	31	22	45	7.8%	1.12 [0.45, 2.79]
Kuo *et al*[41]	58	83	53	83	9.1%	1.31 [0.69, 2.51]
Subtotal (95% CI)		284		293	49.3%	1.27 [0.70, 2.31]
Total events	203		190			
Heterogeneity: Tau^2 = 0.35; χ^2 = 13.88, df = 6 (P = 0.03); I^2 = 57%						
Test for overall effect: Z = 0.79 (P = 0.43)						
Total (95% CI)		723		608	100.0%	1.59 [0.98, 2.58]
Total events	553		410			
Heterogeneity: Tau^2 = 0.57; χ^2 = 41.66, df = 13 (P < 0.0001); I^2 = 69%						
Test for overall effect: Z = 1.86 (P = 0.06)						
Test for subgroup differences: χ^2 = 0.77, df = 1 (P = 0.38); I^2 = 0%						



B

Study or subgroup	LA-7 Events	Total	Quadruple therapy Events	Total	Weight	Odds ratio M-H, random, 95% CI
1.1.1 Caucasian						
Nista *et al*[21]	0	0	44	70		Not estimable
Perri *et al*[29]	37	58	46	55	12.4%	0.34 [0.14, 0.84]
Orsi *et al*[38]	0	0	44	50		Not estimable
Bilardi *et al*[39]	0	0	6	17		Not estimable
Nista *et al*[35]	0	0	25	35		Not estimable
Nista *et al*[36]	37	50	34	50	12.6%	13.34 [0.56, 3.19]
Gisbert *et al*[28]	0	0	21	38	24.9%	Not estimable
Subtotal (95% CI)		108		315		0.68 [0.18, 2.58]
Total events	74		220			
Heterogeneity: Tau^2 = 0.72; χ^2 = 4.57, df = 1 (P = 0.03); I^2 = 78%						
Test for overall effect: Z = 0.56 (P = 0.57)						
1.1.2 Asian						
Wong *et al*[44]	21	33	22	30	10.7%	0.64 [0.22, 1.87]
Lee *et al*[80]	5	9	4	14	6.3%	3.13 [0.54, 18.04]
Wong *et al*[19]	19	31	26	30	9.1%	0.24 [0.07, 0.87]
Zhang *et al*[43]	42	49	30	44	11.2%	2.80 [1.01, 7.77]
Zhang *et al*[37]	42	48	33	47	10.9%	2.97 [1.03, 8.57]
Jung *et al*[42]	16	31	22	45	12.1%	1.12 [0.45, 2.79]
Kuo *et al*[41]	58	83	53	83	14.7%	1.31 [0.69, 2.51]
Subtotal (95% CI)		284		293	75.1%	1.27 [0.70, 2.31]
Total events	203		190			
Heterogeneity: Tau^2 = 0.35; χ^2 = 13.88, df = 6 (P = 0.03); I^2 = 57%						
Test for overall effect: Z = 0.79 (P = 0.43)						
Total (95% CI)		392		608	100.0%	1.09 [0.63, 1.87]
Total events	277		410			
Heterogeneity: Tau^2 = 0.41; χ^2 = 21.29, df = 8 (P = 0.006); I^2 = 62%						
Test for overall effect: Z = 0.31 (P = 0.75)						
Test for subgroup differences: χ^2 = 0.70, df = 1 (P = 0.40); I^2 = 0%						



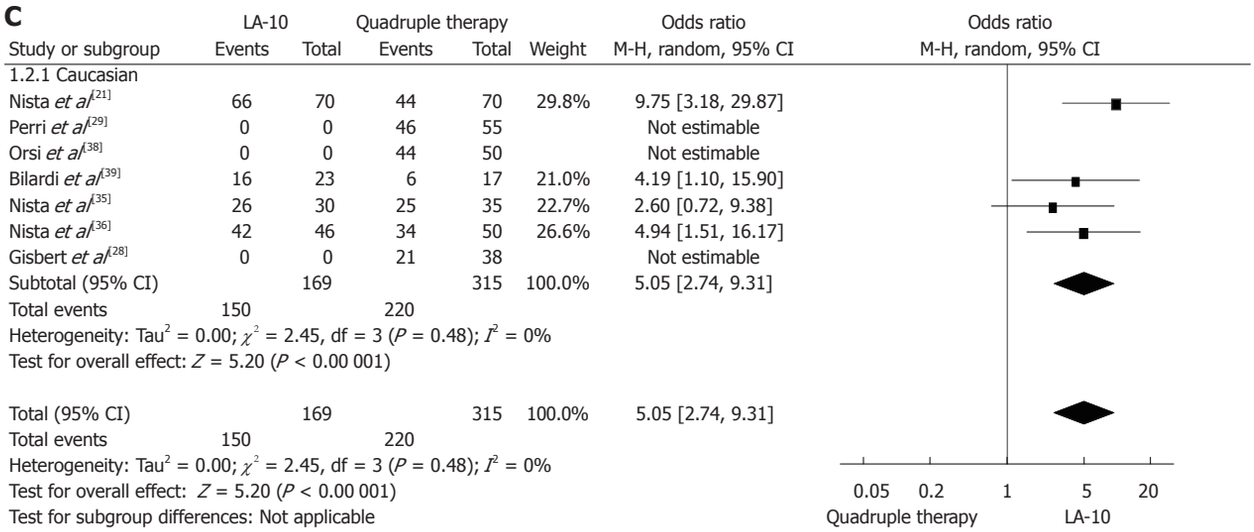


Figure 1 Meta-analysis comparing the efficacy of anti-*Helicobacter pylori* levofloxacin/amoxicillin-based triple therapy *vs* quadruple therapy 7-10 or 12 d (A), 7 d (B), 10 d (C) levofloxacin/amoxicillin regimens were considered. CI: Confidence interval; LA: Levofloxacin/amoxicillin.

resistance testing is not routinely performed to guide prescription. Therefore, even if widely used, published data on levofloxacin-based regimens for *H. pylori* eradication are extremely heterogeneous. Differences in dosages, length of treatment, drug combination, patients' demographic characteristics, and previous courses of therapy, preclude a definitive conclusion.

The present study included only studies comparing cure rate and tolerability of standard QT regimen to 7 or 10 d combination of LA-based triple therapy in second-line, to provide guidance in clinical practice. In accordance with previous reports[70-72], our data showed that 7 d LA-based triple regimen and QT achieved similar efficacy results, whereas LA-based regimen administered for 10 d was more effective than QT. Thus, tolerability was optimal in LA-based triple therapy and patients experienced a lower rate of adverse events compared to patients treated with standard QT, with the exception of a single discordant study[28]. However, in a multicenter study, the same author reported remarkably superior tolerability using a 10-d levofloxacin-based regimen[38,40].

In previous studies, dose of quinolone appeared the crucial factor influencing the incidence of side effects[70,71,73]. In most studies included in the analysis, levofloxacin has been administered at a dosage of 500 mg daily. Interestingly, a higher incidence of side effects was registered in one of the trials using 1 g in two divided doses[28].

Statistical comparison of side effects experienced in 7-d *vs* 10-d groups was not consistent due to the structure of the selected studies. With the exception of the study by Gisbert *et al*[28], tolerability was excellent in all other trials using levofloxacin-based regimens for 10 d or longer. Probably, acceptance of LA therapy depends on the simplicity of the therapeutic regimen itself. Although a new preparation of single three-in-one capsules containing bismuth subcitrate potassium, metronidazole, and tetracycline has been recently proposed in first-line[74], QT still consists of a complex scheme (12 pills/d), and bismuth salts are no longer available in many countries. On the contrary, levofloxacin-based therapy appears simple (5 pills/d), effective (10-d regimen eradication rate: 88.7%) and safe (overall side effects incidence: 13.7%). Duration of treatment appeared the unique factor influencing the efficacy[75].

Nevertheless, considering that the ideal for *H. pylori* infection might differ between areas, countries and races[76-78], we performed an additional analysis on efficacy in different parts of the world.

In a sub-analysis comparing Asian and European trials, QT was equally effective, while LA-based regimens were more effective in the European population. Differences in efficacy rates between Asian and European populations using LA-based schemes might be explained with primary antibiotic resistance and/or genetic background. The meta-analysis showed that the LA-based regimen was more effective in Caucasians only when any regimen was included. However, 10-d levofloxacin-based treatment has never been tested in Asian countries and efficacy increases with duration of treatment, therefore, it is difficult to establish whether differences reported depended on ethnicity or duration of therapy.

Regarding incidence of side effects, ethnicity appeared to affect tolerability of QT compared with LA-based therapy in Asian populations. However, the single outlier among European trials might bias this finding.

Apart from levofloxacin, other regimens containing fluoroquinolones have been proposed both in first- and second-line *H. pylori* treatment. Among these, moxifloxacin-based regimens appear interesting (eradication rate up to 90% and 70%, respectively in first and second-line[72,75,79]. With respect to levofloxacin, moxifloxacin has been relatively more recently introduced on the market, with a consequent possibly lower rate of antibiotic resistance. However, it is more expensive. Nevertheless, no conclusive recommendations can be issued on which

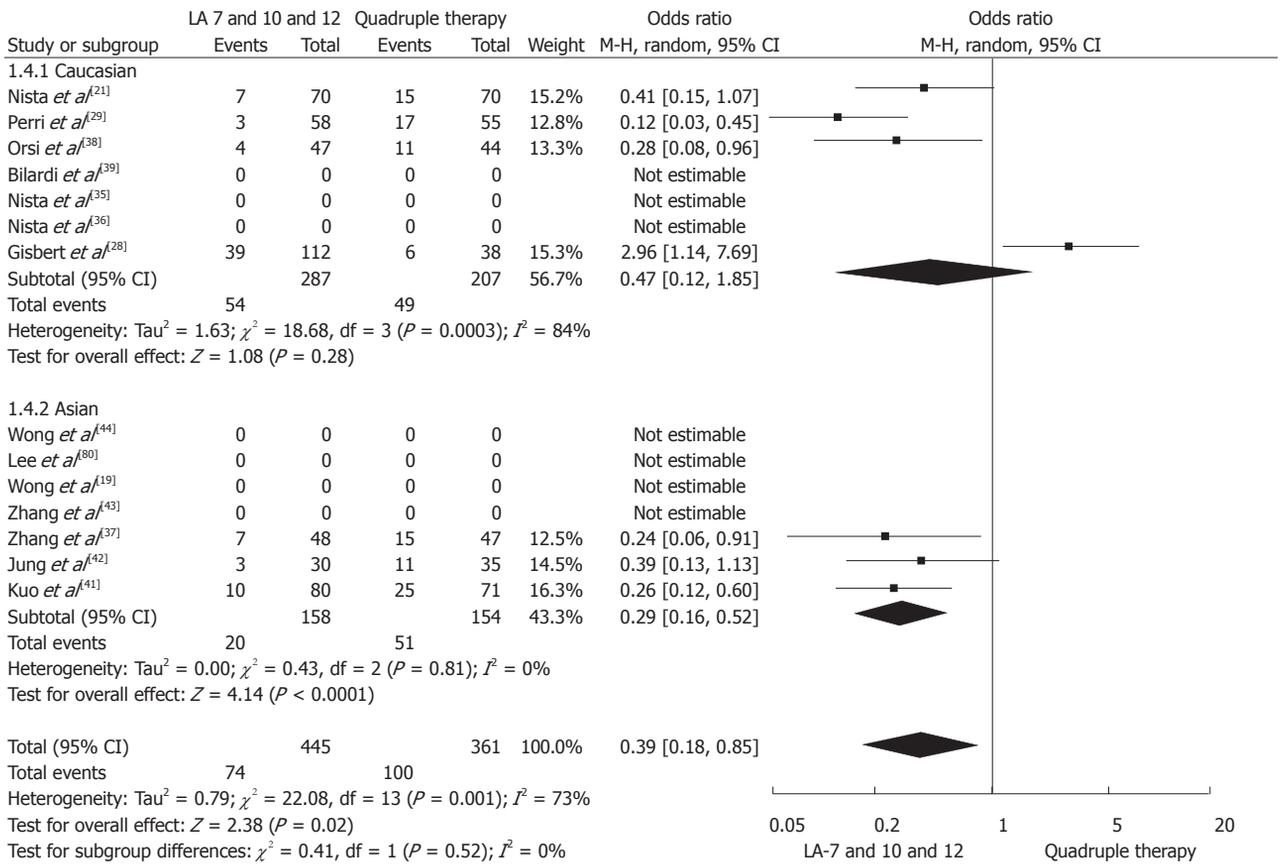


Figure 2 Meta-analysis comparing the incidence of side effects with levofloxacin/amoxicillin-based triple therapy *vs* quadruple therapy for *Helicobacter pylori* eradication. CI: Confidence interval; LA: Levofloxacin/amoxicillin.

fluoroquinolone should be used because there are no trials comparing the two drugs.

Our results are in accordance with previous meta-analyses assessing efficacy and safety of second-generation fluoroquinolones, including levofloxacin and moxifloxacin[13,71,72,79], for *H. pylori* eradication. However, compared to previous studies, our study included the most up to date collection of RCTs and most importantly, compared exclusively second-line treatments and analyzed geographical stratification.

Our meta-analysis had several limitations, mainly due to heterogeneity among trials (different dosing schedules and duration of treatment). Sub-analyses were mainly affected by the small sample size for each category. Finally, the lack of data available on use of 10 d LA-based therapy in Asian populations did not allow a definitive conclusion about the influence of ethnicity on treatment success. Moreover, considering the suboptimal tolerance of QT in Asian populations, alternative strategies, such as 10 d LA-based therapy, and randomized trials in this geographic setting would be desirable.

In our analysis, we selected exclusively RCTs comparing LA-based triple regimens to standard QT to provide practical useful data on a specific treatment to use as rescue regimen of choice due to the rare *H. pylori* resistance to amoxicillin.

In conclusion, this meta-analysis strongly supports the use of 10 d LA-based triple regimen as a simple (5 pills/d) second-line treatment for *H. pylori* eradication, with an excellent eradication rate and tolerability profile in comparison to standard recommended QT in Asian and European countries. Nevertheless, quinolone resistance monitoring in different geographical areas is required.

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REVIEW

Multimodality approach for locally advanced esophageal cancer

Khaldoun Almhanna, Jonathan R Strosberg

Khaldoun Almhanna, Jonathan R Strosberg, Department of Gastrointestinal Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, United States
Author contributions: Both authors contributed equally to the manuscript.
Correspondence to: Jonathan R Strosberg, MD, Department of Gastrointestinal Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, United States. jonathan.strosberg@moffitt.org
Telephone: +1-813-7457257 Fax: +1-813-7457229
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Abstract

Carcinoma of the esophagus is an aggressive and lethal malignancy with an increasing incidence worldwide. Incidence rates vary internationally, with the highest rates found in Southern and Eastern Africa and Eastern Asia, and the lowest in Western and Middle Africa and Central America. Patients with locally advanced disease face a poor prognosis, with 5-year survival rates ranging from 15%-34%. Recent clinical trials have evaluated different strategies for management of locoregional cancer; however, because of stage migration and changes in disease epidemiology, applying these trials to clinical practice has become a daunting task. We searched Medline and conference abstracts for randomized studies published in the last 3 decades. We restricted our search to articles published in English. Neoadjuvant chemoradiotherapy followed by surgical resection is an accepted standard of care in the United States. Esophagectomy remains an essential component of treatment and can lead to improved overall survival, especially when performed at high volume institutions. The role of adjuvant chemotherapy following curative resection is still unclear. External beam radiation therapy alone is considered palliative and is typically reserved for patients with a poor performance status.

INTRODUCTION

Carcinoma of the esophagus is an aggressive disease associated with a poor overall survival (OS). In 2011, 16 980 patients will be diagnosed in the United States, and 14 710 will die from their disease[1]. The incidence of adenocarcinoma of the esophagus is rising dramatically worldwide, while the incidence of squamous cell carcinoma (SCC) is decreasing[2]. While survival rates in metastatic esophageal cancer remain low, outcomes among patients with resectable locoregional disease have improved with the incorporation of multimodality treatment including chemotherapy, radiation and surgical resection. Multiple clinical trials have addressed the preferred treatment sequence in managing locally advanced esophageal cancer (LAEC); however, no standard therapy has been established.

Esophagectomy remains the cornerstone treatment for early-stage esophageal carcinoma; however surgery alone is rarely curative for tumors invading beyond the muscularis propria or involving locoregional lymph nodes. As a result, many clinical trials have addressed the role of chemotherapy and radiation in the neoadjuvant or adjuvant setting, with conflicting results.

The majority of the studies published in the last three decades included mostly patients with SCCs, and a distinction between the two subtypes was not always made. However, it remains unclear as to whether histology should dictate treatment approach. Extrapolation from old literature is acceptable until further data in adenocarcinoma is available. Enrollment in clinical trials should be encouraged.

In this article we will review the literature on the treatment of locally advanced mid-distal esophageal cancer. Treatment recommendations for cervical esophageal cancer will not be included in this review.

TREATMENT WITH SURGERY ALONE *VS* RADIOTHERAPY ALONE

Surgical resection remains the standard treatment for early-stage disease. Endoscopic mucosal resection (EMR) is recommended for T1a tumors if negative margins can be achieved. Esophagectomy upfront is recommended in patients with positive margins following EMR, or in patients with T1b disease.

Surgery alone as a treatment option for esophageal cancer was found to be superior when compared to radiation therapy. In a randomized trial of patients with LAEC, 39 patients were randomized to surgery and 35 patients to 45 Gy to 53 Gy radiation over four to five weeks[3]. The median survival for surgery was 21.6 mo compared with 8.2 mo for the radiation only group. In another trial comparing 47 surgical patients to 52 patients undergoing radiation[4], OS was improved with surgery [odds ratio (OR) 2.74].

Despite innovations in surgical techniques and declines in perioperative mortality, 5-year survival rates rarely exceed 50% among patients with stage Ⅱ or higher disease[5-7]. Median overall and disease-free survival does not appear to differ significantly based on the surgical approach (transthoracic *vs* transhiatal resection)[5]. In recent years, mortality rates after esophagectomy have decreased significantly. High-volume institutions have superior operative mortality rates (more than 12% absolute reduction in mortality when compared to low volume centers)[8].

PREOPERATIVE RADIOTHERAPY *VS* SURGERY ALONE

Radiotherapy as a single modality has been evaluated as a treatment option for esophageal cancer. In a small trial of 17 patients with clinical stage I esophageal SCC treated with radiation alone, the five-year survival rate was 59%[9]. In another single-institution series of 101 patients with LAEC treated with radiotherapy alone, the five-year survival rate was 21%. Survival was superior for adenocarcinoma compared to SCC, however the difference was not statistically significant[10].

Several randomized trials comparing neoadjuvant radiotherapy and surgery *vs* surgery alone in patients with LAEC have been reported[3,11-15]. Investigators used different radiation doses and techniques. There was no statistically significant difference in OS with preoperative radiotherapy compared with surgery alone.

In a trial by Launois *et al*[11], 124 patients with SCC of the esophagus were randomized to receive preoperative radiation for eight days *vs* surgery alone. No significant differences in outcome were noted between the two groups. Of the 62 irradiated patients, 14 died during operation, compared with 11 of the 47 non-irradiated patients, which was not statistically significant. 5-year survival rates were similarly poor (9.5% *vs* 11.5%).

In 1987, Gignoux *et al*[12] from the European Organization for Research on Treatment of Cancer (EORTC) compared surgery alone to 33 Gy of preoperative radiation therapy followed by surgery. Although local disease control was improved, there was no difference in survival or resectability of tumors.

Another prospective randomized clinical trial (RCT) randomized patients to preoperative radiation followed by surgery (104 patients) *vs* surgery along (102 patients)[13]. No significant differences were reported between the two groups in resection rates, operative mortality, anastomotic leaks, positive margins or lymph nodes metastases. The 5-year survival rates in the combined modality treatment group and the surgery alone group were 35% and 30% respectively.

In a four-arm Scandinavian study of patients with non-metastatic squamous cell esophageal carcinoma[14], patients were randomized to surgery alone, preoperative radiation, preoperative chemotherapy, or preoperative chemoradiation. Although the three-year survival was significantly higher in the pooled groups receiving radiotherapy as compared with the pooled groups not receiving radiotherapy, patients who received preoperative radiation alone *vs* surgery alone had a median survival of 10 mo *vs* 7 mo respectively which was not statistically significant.

In a different study, 176 patients with potentially operable SCC or adenocarcinoma of the esophagus were randomly assigned to preoperative radiotherapy or surgery alone[15]. Preoperative radiotherapy was not associated with any significant operative complications. The median OS for the entire cohort was 8 mo, and the 5-year survival was 13%. There was no significant difference in survival between the two groups. Trials of preoperative radiation are summarized in Table 1.

A meta-analysis of 1147 cases, predominantly SCC, from five RCTs examining preoperative radiotherapy and surgery compared to surgery alone[16] failed to demonstrate a conclusive survival benefit in LAEC. The hazard ratio (HR) associated with neoadjuvant radiotherapy was 0.89 (95% CI: 0.78-1.01; $P = 0.06$).

POSTOPERATIVE RADIOTHERAPY *VS* SURGERY ALONE

In order to reduce the risk of local recurrence, several randomized trials have compared surgery alone to sur-

Table 1 Randomized trials of preoperative radiotherapy *vs* surgery alone

Ref.	Number of patients	Median survival (mo)		5-yr survival[1] (%)	
		+RT	-RT	+RT	-RT
Launois *et al*[11]	124	4.5	8.2	10	12
Gignoux *et al*[12]	229	12.3	12	10	9
Wang *et al*[13]	206	-	-	35	30
Nygaard *et al*[14]	108	10	7	-	-
Arnott *et al*[15]	196	8	8	9	17
Fok *et al*[3]	79	11	22	10	16

[1]Not statistically significant. RT: Radiotherapy.

gery followed by adjuvant radiation therapy. Studies enrolled a heterogonous population including patients with positive celiac nodes[17,18] and R1 resections[19]. Investigators used varying doses of radiation with different fractionations. Radiation was delivered from 6-12 wk following surgery.

These trials had conflicting results: three failed to demonstrate any survival benefits with adjuvant radiation[17,18,20], while one trial showed inferior survival among patients who received adjuvant radiation therapy[19]. One trial showed improvement in quality of life in the surgery-only arm[18] compared to the adjuvant radiation arm. A pooled analysis of the five adjuvant radiation trials[21] showed no significant difference in survival following adjuvant radiotherapy when compared to surgery alone. A reduction in local recurrence was reported in some of these trials at the expense of toxicity. Preoperative radiotherapy was compared to postoperative radiotherapy in a small randomized trial; no difference in survival was detected between the two arms but patients receiving preoperative radiotherapy experienced higher morbidity[3].

PREOPERATIVE CHEMOTHERAPY *VS* SURGERY ALONE

Multiple randomized trials compared preoperative chemotherapy to surgery alone for the treatment of resectable esophageal cancer. Of the seven trials summarized in Table 2, four showed no survival benefit while three were positive demonstrating improved survival with neoadjuvant chemotherapy. Roth *et al*[22] randomized 39 patients with potentially resectable cancer of the middle or lower esophagus to receive either surgery along, or preoperative and postoperative therapy with cisplatin, vindesine, and bleomycin. The overall resectability rates were similar for the two groups. Patients responding to chemotherapy preoperatively had a significantly prolonged survival (greater than 20 mo) when compared with either nonresponders (median 6.2 mo) or patients in the surgical monotherapy arm (median 8.6 mo).

In a phase Ⅲ study[23], forty six patients with potentially resectable SCC of the esophagus were randomized to undergo either immediate surgery (n = 24) or surgery plus preoperative chemotherapy (n = 22). Chemotherapy consisted of three cycles with cisplatin and 5-fluorouracil (5-FU). The response rate to chemotherapy was 50% (17 of 34 patients). Side effects of therapy were higher than expected based on results of previous phase Ⅱ studies. Two drug-related deaths were observed. The resectability rate for patients in the surgery only group was 79% compared with 70% for patients receiving preoperative chemotherapy. Patients who responded to preoperative chemotherapy had a prolonged median survival (13 mo) compared with nonresponders (5 mo), but the median survival for the chemotherapy group and the surgery only group was identical (10 mo).

In a later study, Law *et al*[24] randomized 147 patients to receive surgery alone or preoperative cisplatin and 5-FU. Of the 60 patients in the preoperative chemotherapy arm, 58% had a significant response. Curative resections were possible in 67% of these patients compared with 35% in the control group. Median survivals were 16.8 and 13 mo, respectively (P = 0.17).

In a randomized phase Ⅲ trial[25], 42 patients were randomized to receive either two cycles of chemotherapy (cisplatin, bleomycin, 5-FU and vinblastine) followed by surgery or surgery alone. Median survival time was 17 mo in both groups. However, early survival appeared to be better in the control group.

Larger, more recent studies have shown improved benefits for patient receiving neoadjuvant therapy. In the medical research council study[26], 802 patients with resectable esophageal cancer were randomly allocated either two cycles of cisplatin and 5-FU followed by surgical resection or resection alone.

Postoperative complications were similar in both groups. OS was prolonged in the chemotherapy group with a median survival of 16.8 mo compared with 13.3 mo in the surgery only group. In the Medical Research Council adjuvant gastric infusional chemotherapy trial[27], 503 patients with resectable adenocarcinoma of the stomach, esophagogastric junction, or lower esophagus were randomly assigned to either perioperative chemotherapy or surgery alone. Chemotherapy consisted of three preoperative and three postoperative cycles of intravenous epirubicin, cisplatin and continuous intravenous infusion of 5-FU. With a median follow-up of four years, OS was significantly improved in the perioperative chemotherapy arm (36% *vs* 23%).

Several pooled and meta-analyses addressed the role of neoadjuvant chemotherapy followed by surgery compared to surgery alone. Malthaner *et al*[21] included five studies in a meta-analysis[14,23-26,28]. No significant difference in outcome was detected (relative risk ratio, 1.00; 95% CI: 0.83-1.19; P = 0.98).

Another meta-analysis was published in 2002 and included eleven RTCs comparing surgery alone to preoperative chemotherapy[29]. Again, there was no statistically significant difference in 3-year survival rates among patients who received preoperative chemotherapy com-

Table 2 Randomized trials of preoperative chemotherapy *vs* surgery alone

Ref.	Number of patients	Chemotherapy	Median survival (mo)		3-yr survival (%)	
			+CT	-CT	+CT	-CT
Roth JA *et al*[22]	39	CDDP/bleomycin/vindesin	> 20[1]	8.6	-	-
Nygaard *et al*[14]	112	CDDP/bleomycin	7	7	3	9
Schlag[23]	46	CDDP/5-FU	7.5	5	-	-
Maipang *et al*[25]	46	CDDP/bleomycin	17	17	31	36
Law *et al*[24]	147	CDDP/5-FU	16.8	13	38	14
Kelsen *et al*[56]	440	CDDP/5-FU	14.9	16.1	42	45
MRC[26]	802	CDDP/5-FU	16.8	13.3	35	27
Ancona *et al*[28][1]	94	CDDP/5-FU	25	24	44	41
Cunningham *et al*[27][1]	503	ECF	-	-	79	50

[1]The difference in survival was statistically significant. MRC: Medical Research Council Oesophageal Cancer Working Group; CDDP: Cisplatin; 5-FU: 5-flurouracil; ECF: Epirubicin, cisplatin and 5-fluorouracil; CT: Chemotherapy.

pared to those who did not.

Two other meta-analyses showed survival benefits with preoperative chemotherapy; the first was a Cochrane Review which pooled 11 RTCs. A statistically significant difference in survival favouring preoperative chemotherapy was detected at five years[30]. A more updated meta-analysis[31] comparing survival with or without neoadjuvant chemotherapy in 9 RTCs (total of 1981 patients) found that chemotherapy was associated with a significant survival benefit (pooled HR of 0.87; $P = 0.005$) corresponding to an absolute survival difference at 2 years of 5.1%.

POSTOPERATIVE CHEMOTHERAPY *VS* SURGERY ALONE

A small number of randomized trials have evaluated postoperative chemotherapy compared with surgery alone. Pouliquen *et al*[32] found no improvement in survival rates with postoperative chemotherapy consisting of cisplatin and 5-FU in patients with SCC. Duration of dysphagia was similar for both groups as well. Another randomized trial compared surgery alone *vs* surgery followed by adjuvant cisplatin and 5-FU in 242 Japanese patients with SCC[33]. Disease free survival at 5 years favoured the adjuvant therapy arm (55% *vs* 45%), however, improvement in OS did not reach statistical significance (61% *vs* 52%). Despite the short duration of adjuvant chemotherapy, 25% of patients in the chemotherapy arm failed to receive the full course of adjuvant therapy.

PREOPERATIVE *VS* POSTOPERATIVE CHEMOTHERAPY

A small number of randomized trials have evaluated postoperative chemotherapy compared to preoperative chemotherapy. A recent Japanese study[34] randomized 330 patients with SCC of the esophagus to surgery followed or preceded by two courses of cisplatin plus 5-FU. The primary end point was progression-free survival. Progression-free survival did not reach the stopping boundary, but OS in the preoperative group was superior to that of postoperative group. The study was published early and concluded that preoperative chemotherapy with cisplatin plus 5-FU can be regarded as standard treatment. In a prior trial[33], PFS was better in patients who received postoperative *vs* preoperative chemotherapy, but the OS was not different for the two groups. Patients with node-negative cancer did not benefit from postoperative chemotherapy.

Concurrent chemotherapy and radiation

Concurrent chemoradiation has been tested in LAEC as neoadjuvant, adjuvant or definitive therapy in order to optimize local and systemic disease control.

Definitive chemoradiation: Definitive chemoradiation has been studied in primarily in locally advanced, squamous cell unresectable esophageal cancer. RTOG 85-01[35] was a pivotal trial which compared radiation alone *vs* concurrent chemoradiation (cisplatin and 5-FU) in patients with LAEC, ninety percent of whom had SCC. The chemoradiotherapy group received two additional chemotherapy cycles following radiation.

An interim analysis showed a significant benefit with chemoradiation with a five-year survival of 27% compared to 0 %[36]. However, 46% of patients in the chemoradiation arm had locally recurrent or persistent disease in the esophagus which lead to the intergroup 0123[37] trial where patients with LAEC were randomized to two different radiation doses concurrent with chemotherapy (50.4 gray *vs* 64.8 gray). A higher dose was not associated with improved locoregional control.

Preoperative chemoradiation: The poor outcome associated with surgery alone and the high locoregional recurrence with definitive chemoradiation provided the rational for evaluating neoadjuvant chemoradiation followed by surgery in patients with resectable esophageal cancer.

More than ten randomized trials compared neoadjuvant chemoradiation followed by surgery to other treatment modalities. These trials are summarized in Table 3.

Table 3 Randomized trials of preoperative chemotherapy and radiation *vs* surgery alone

Ref.	Number of patients	Chemotherapy	Median survival (mo)		3-yr survival (%)	
			+CRT	-CRT	+CRT	-CRT
Nygaard *et al*[14]1	103	CDDP/bleomycin	7	7	17	9
Le Prise *et al*[39]	86	CDDP/5-FU	11	11	19	14
Apinop *et al*[40]	69	CDDP/5-FU	9.7	7.4	26	20
Walsh *et al*[47]1	113	CDDP/5-FU	16	11	32	6
Bosset *et al*[38]	282	CDDP	18.6	18.6	39	37
Urba *et al*[46]	100	CDDP/vinblastine/5-FU	17.6	16.9	30	16
Burmeister *et al*[43]	257	CDDP/5-FU	22	19	-	-
Lee *et al*[48]	101	CDDP/5-FU	28.2	27.3	-	-
Tepper[45]1	56	CDDP/5-FU	53	21	-	-
van Hagen[41]1	386	Carbo/paclitaxel	49	24	58	44

[1]The difference in survival was statistically significant. CDDP: Cisplatin; 5-FU: 5-flurouracil; CRT: Chemotherapy and radiation.

Four trials were conducted with preoperative sequential chemotherapy and radiation and 8 trials were performed with chemoradio-therapy. The sequential trials were restricted to SCC and all but three of the concurrent trials included patients with adenocarcinoma. None of the four trials of sequential therapy showed a survival benefit[14,38-40]. However, the combined radiation arms in the second Scandinavian trial showed a survival benefit compared to the non-radiation arms[14]. Additionally, chemotherapy was not associated with a survival benefit. In the EORTC trial, while preoperative therapy was associated with longer disease-free survival, longer interval of local control, and lower rates of cancer-related deaths, postoperative deaths were higher and were attributed to the hypofractionated radiation regimen used[38]. Of the 8 randomized trials of neoadjuvant chemoradiation[41-49], 4 showed a survival benefit from[41,45,47,49]. In the Irish study, 113 patients were randomized to 40 Gy in 3 wk concurrent with cisplatin and 5-FU followed by surgery *vs* surgery alone[47]. The trial resulted in a significant survival benefit with a median and 3 year survival of 16 mo and 32% for the chemoradiation group *vs* 11 mo and 6% for the surgery only group ($P = 0.01$). This trial was highly criticized for the low survival in the surgery only arm compared to other studies and for short follow up. Tepper *et al*[45] intended to randomize 475 patients to neoadjuvant chemoradiotherapy with 50.4 Gy over 5.5 wk concurrent with cisplatin and 5-FU and surgery *vs* surgery alone in a Cancer and Leukemia Group B study, however the trial was closed due to poor accrual. Only 56 patients were enrolled. Median and 5 year survival was 4.5 years and 39% for chemoradiation patients *vs* 1.8 years and 16% for surgery only patients ($P = 0.02$). The study was criticized for being underpowered. While the Michigan study showed a near doubling of 3-year survival in chemoradiation patients, the study was also underpowered to detect a significant difference. The study was powered to detect an increase in median OS from 1 year to 2.2 years[46]. A recently published phase Ⅲ study (the study "Effect of preoperative concurrent chemoradiotherapy on survival of patients with resectable esophageal or esophagogastric junction cancer")[41] randomized patients with resectable esophageal cancer to receive surgery alone or weekly administration of carboplatin and paclitaxel for 5 wk and concurrent radiotherapy followed by surgery. Seventy-five percent of patients had adenocarcinoma. R0 resection was achieved in 92% of patients in the chemoradiotherapy-surgery group *vs* 69% in the surgery group ($P < 0.001$). A pathological CR was achieved in 29% of patients who underwent resection after chemoradiotherapy. Postoperative complications were similar in both groups as was in-hospital mortality. Median OS was 49.4 mo in the chemoradiotherapy-surgery group *vs* 24.0 mo in the surgery group (HR 0.657; 95% CI: 0.495-0.871; $P = 0.003$).

A three arm study from China, where patients with SCC were randomized to preoperative chemoradiation, postoperative chemoradiation, or surgery alone, showed a survival benefit for both preoperative and postoperative chemoradiation. A recent French trial (FFCD9901) determined the effect of preoperative chemoradiation in stage I and II esophageal cancers (71% SCC)[44]. There was no difference in survival and postoperative 30 d mortality rates were trending higher in chemoradiation patients (7.3% *vs* 1.1%; $P = 0.054$).

The majority of patients included in these studies had SCC. Clinical staging was suboptimal by modern standards. An Irish[47] and Dutch[41] trial each showed statistically significant improvement in OS with combined preoperative chemoradiation but the Irish study was criticized for the lack of appropriate staging, premature closure, and an unusually poor survival rate in the surgery-alone arm.

Several meta-analyses have addressed the benefit of trimodality therapy over surgery alone for esophageal cancer. The first, by Fiorica *et al*[49] pooled six RCTs comparing preoperative chemoradiation and surgery *vs* surgery alone. Chemotherapy and radiation followed by surgery significantly decreased mortality (OR 0.53; 95% CI: 0.31-0.92; $P = 0.03$). However, the risk of postoperative mortality was higher in the multimodality arm.

A second meta-analysis, by Urschel *et al*[50], pooled nine RCTs including 1116 patients. A statistically significant survival difference was found at three years in

favor of preoperative chemoradiation (OR 0.66; 95% CI: 0.47-0.92; $P = 0.016$). Neoadjuvant chemoradiation was associated with a lower rate of resection, but a higher rate of complete (R0) resection. There was a non-significant trend toward increased treatment mortality with neoadjuvant chemoradiation.

A third meta-analysis by Gebski *et al*[51] pooled 10 RTCs comparing neoadjuvant chemoradiotherapy *vs* surgery alone with a total of 1209 patients. A significant survival benefit was evident for preoperative chemoradiotherapy with a HR of 0.81 (0.70-0.93; $P = 0.002$), corresponding to a 13% absolute difference in survival at 2 years. The HR was 0.84 (0.71-0.99; $P = 0.04$) for SCC and 0.75 (0.59-0.95; $P = 0.02$) for adenocarcinoma.

A fourth meta-analysis by Jin *et al*[52] pooled 11 RTCs comparing neoadjuvant chemoradiotherapy *vs* surgery alone with a total of 1308 patients. A significant survival benefit was evident for preoperative chemoradiotherapy at 3 years with an OR of 1.78 (1.20-2.66; $P = 0.004$). In a sub-group analysis, SCC patients did not benefit from neoadjuvant chemoradiotherapy.

The most recent meta-analysis by Sjoquist *et al*[31] included 12 RTCs of neoadjuvant chemoradiotherapy *vs* surgery alone. The HR for all-cause mortality with neoadjuvant chemoradiotherapy was 0.78 (95% CI: 0.70-0.88; $P < 0.0001$); the HR for SCC was 0.80 (95% CI: 0.68-0.93; $P = 0.004$) and for adenocarcinoma was 0.75 (95% CI: 0.59-0.95; $P = 0.02$). This analysis also compared neoadjuvant chemoradiation *vs* chemotherapy and demonstrated a non-statistically-significant survival benefit for neoadjuvant chemoradiation (HR 0.88, 95% CI: 0.76-1.01; $P = 0.07$).

Postoperative chemoradiation: There are no randomized trials demonstrating that chemoradiation benefits patients who did not receive neoadjuvant therapy for LAEC. The role of adjuvant therapy is particularly uncertain in patients who have positive margins or node positive disease. Based on the intergroup 0116 trial[53] of patients with gastric cancer [20% of whom had gastro-esophageal junction (GEJ) adenocarcinoma], patients often receive adjuvant chemoradiation following resection; however toxicities associated with administration of chemoradiation after esophagectomy (e.g., pneumonitis) can be significant.

One randomized trial evaluated postoperative chemoradiation *vs* postoperative radiotherapy following esophagectomy. No difference in survival was detected (38 mo for both groups)[3]. Toxicity was higher in the combined treatment arm.

NEOADJUVANT CHEMOTHERAPY *VS* NEOADJUVANT CHEMO-RADIATION

Two randomized trials compared neoadjuvant chemotherapy to neoadjuvant chemoradiation. Both trials included patients with adenocarcinoma, were closed early, and were underpowered to show a survival advantage.

In an Australian trial[54], 75 patients were randomized to receive preoperative cisplatin and 5-FU or preoperative chemoradiation with the same drugs at lower doses. The pathological response rate was higher in the combination arm (31% *vs* 8%; $P = 0.01$). The median progression-free survival was 26 mo in the combination arm *vs* 14 mo in the chemotherapy arm ($P = 0.37$). The median OS was 29 mo *vs* 32 mo for the combination arm but did not reach statistical significance ($P = 0.83$).

In the German phase Ⅲ Preoperative Chemotherapy or Radiochemotherapy in Esophago-gastric Adenocarcinoma trial[54,55], 126 patients with GEJ adenocarcinoma were randomly assigned to neoadjuvant chemotherapy alone (cisplatin and 5-FU) for 16 wk *vs* 12 wk of the same regimen followed by low-dose radiotherapy concurrent with cisplatin and etoposide. The pathologic complete remission rate was significantly higher in the induction arm (16% *vs* 2%), with a non-significant trend towards superior three-year survival as well (47% *vs* 28%; $P = 0.07$). A meta-analysis of the two trials[31] favored neoadjuvant chemoradiation but this was not statistically significant.

DISCUSSION

Esophageal cancer is not a common disease with less than 20 000 cases diagnosed in the US every year. Several clinical trials have evaluated different modalities for management of locoregional cancer; however, large phase Ⅲ trials comparing chemotherapy, radiation, surgery or any combination are lacking. Clinical trials reviewed in this article included heterogeneous groups of patients with adenocarcinoma and SCC without uniform staging studies. Stage migration and changes in disease epidemiology impede application of these trials to clinical practice. Comparing outcome (survival or progression free survival) across clinical trials is not recommended in oncology in general and especially in esophageal cancer as the changes in outcome might not be related to the different chemotherapy agents or radiation techniques as much as disease pathology, patient selection and changes in surgical outcomes across more than three decades.

Accumulating evidence suggests that patients whose tumors have invaded beyond the submucosa (T2 or above) and who are surgical candidates should undergo some form of neoadjuvant therapy including chemotherapy, radiation or both prior to surgical resection. Although controversial, esophagectomy remains an essential part of treatment and can lead to improved OS, especially when performed in a high-volume institution. Even in patients with SCC, where definitive chemoradiotherapy is a potentially curative, there remains a high rate of local failure after chemoradiotherapy alone. We therefore recommend surgical resection in all patients with mid to distal esophageal cancer who are surgical candidates. Based on the above trials and meta-analyses, we generally administer neoadjuvant chemoradiotherapy rather than neoadjuvant chemotherapy or surgery alone for LAEC. While neoadjuvant chemotherapy provides

a significant survival benefit, local failure rates are still higher than in patients treated with neoadjuvant chemoradiation. Moreover, data on neoadjuvant chemotherapy without radiation are lacking in SCC.

The optimal neoadjuvant regimen has not yet been established. The above mentioned trials used different drugs, doses, and schedules of chemotherapy and radiotherapy. We recommend platinum-based combination chemotherapy rather than a single agent cisplatin.

Following resection, data on adjuvant therapy are lacking. For patients with completely-resected node-positive disease who have not received neoadjuvant therapy, we suggest postoperative adjuvant therapy with chemotherapy and radiation. In patients with residual lymph node positivity following neoadjuvant chemoradiation, long-term recurrence rates are high; however the potential benefits of further adjuvant chemotherapy in this setting are not clear.

Future trials in LAEC should include neoadjuvant chemoradiotherapy as a control arm. While OS should remain a primary endpoint, secondary endpoints should include local disease control and quality of life. Treatment decisions for individual patients should be based on co-morbidities and effects of neoadjuvant therapy on the patient's performance-status and quality of life. Targeted therapies, such as trastuzumab, should be studied in the neoadjuvant and adjuvant settings to improve long-term outcomes.

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REVIEW

Regulatory T cells in inflammatory bowel diseases and colorectal cancer

Györgyi Műzes, Béla Molnár, Ferenc Sipos

Györgyi Műzes, Ferenc Sipos, 2nd Department of Internal Medicine, Semmelweis University, 1088 Budapest, Hungary
Béla Molnár, Molecular Medicine Research Unit, Hungarian Academy of Sciences, 1051 Budapest, Hungary
Author contributions: Műzes G and Sipos F contributed to the writing and editing of this paper; Molnár B revised the manuscript.
Correspondence to: Ferenc Sipos, MD, PhD, 2nd Department of Internal Medicine, Semmelweis University, 1088 Budapest, Hungary. dr.siposf@gmail.com
Telephone: +36-20-4780752 Fax: +36-1-2660816
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Abstract

Regulatory T cells (T_{regs}) are key elements in immunological self-tolerance. The number of T_{regs} may alter in both peripheral blood and in colonic mucosa during pathological circumstances. The local cellular, microbiological and cytokine milieu affect immunophenotype and function of T_{regs}. Forkhead box P3+ T_{regs} function shows altered properties in inflammatory bowel diseases (IBDs). This alteration of T_{regs} function can furthermore be observed between Crohn's disease and ulcerative colitis, which may have both clinical and therapeutical consequences. Chronic mucosal inflammation may also influence T_{regs} function, which together with the intestinal bacterial flora seem to have a supporting role in colitis-associated colorectal carcinogenesis. T_{regs} have a crucial role in the immunoevasion of cancer cells in sporadic colorectal cancer. Furthermore, their number and phenotype correlate closely with the clinical outcome of the disease, even if their contribution to carcinogenesis has previously been controversial. Despite knowledge of the clinical relationship between IBD and colitis-associated colon cancer, and the growing number of immunological aspects encompassing sporadic colorectal carcinogenesis, the molecular and cellular links amongst T_{regs}, regulation of the inflammation, and cancer development are still not well understood. In this paper, we aimed to review the current data surrounding the role of T_{regs} in the pathogenesis of IBD, colitis-associated colon cancer and sporadic colorectal cancer.

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Key words: Regulatory T cells; Forkhead box P3; Inflammatory bowel diseases; Colitis-associated colon cancer; Colorectal cancer

Peer reviewer: Dr. Pingchang Yang, Pathology and Molecular Medicine, McMaster University, BBI-T3330, 50 Charlton Ave East, Hamilton L8N 4A6, Canada

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INTRODUCTION

Immunological self-tolerance is crucial for preventing autoimmune diseases and maintaining immune homeostasis[1]. Regulatory T cells (T_{regs}) are a subpopulation of T cells including CD4+CD25+ forkhead box P3 (Foxp3)+ T cells, Tr1, Th3, and CD8+ T_{regs}. They serve to suppress the immune system and maintain self-tolerance. Inappropriate T cell response to intestinal microflora has a role in the pathological process of inflammatory bowel diseases (IBD)[2]. Accumulating evidence indicates that the imbalance of immunity, including a discrepancy between the number of T_{regs} in peripheral blood and in inflamed colonic mucosa, an excess of pro-inflammatory stimuli and an altered function of immunoregulatory cells has a central role in the pathogenesis of IBD[3].

Risk for developing cancer rises substantially as a result of poorly regulated inflammatory responses to pathogenic bacteria. T_{regs} function to restore immune homeostasis during chronic inflammation, and therefore it seems logical that T_{regs} would reduce the risk of inflammation-associated colon cancer by down-regulating inflammation. Alteration of T cell phenotype could also have an effect not only on the regulation of the chronic mucosal inflammation[3], but on colitis-associated carcinogenesis as well[4,5].

On the other hand, it is also widely believed, that T_{regs} in cancer primarily suppress protective anticancer immune responses. This alteration of T_{regs} function, together with the tumor-helping chemokine milieu produced by the cancer tissue could support cancer progression[4,5]. T_{regs} role in the carcinogenesis of inflammation-associated colon cancer and sporadic colorectal cancer is paradoxical due to conflicting results.

The bone marrow progenitors of all T cells become committed to their lineage in the thymus. The double negative T cells (CD4-CD8-) will rearrange their T cell receptor (*TCR*) genes to form a unique, functional molecule, which they test against cells in the thymic cortex for a minimal level of interaction with self-major histocompatibility complex (MHC). Upon receiving these signals, they begin to proliferate and express both CD4 and CD8. Then, the double-positive cells are selected by their interaction with thymic cells, begin the transcription of Foxp3, becoming T_{regs}. Foxp3 expression begins at the single-positive stage, at which point they are functional T_{regs}[6].

Foxp3 is a member of the forkhead and winged helix family of transcriptional regulators, and plays a critical role in the development and function of CD4+ T_{regs}[6]. In previous studies, CD4 and CD25 were selected as the markers for T_{regs}, but in recent studies Foxp3 has been proposed as a more reliable and superior biomarker[7,8].

The exact process of T_{reg} cell selection is not well known. It appears to be a process determined by the affinity of interaction with the self-peptide MHC complex[9]. Characteristic features of CD4+CD25+Foxp3+ T_{regs} are their anergic state, and their ability to actively inhibit CD4+CD25- T cells, CD8+ T cells, dendritic cells, natural killer cells, natural killer T cells, and B cells in a cell-to-cell contact and dose-dependent manner[10].

Recent results[3-5] suggest that T_{regs} could have an important, but controversial regulatory role in both the pathogenesis of chronic colonic inflammation and colorectal carcinogenesis. Despite the clinical relationship between IBD and IBD-associated colon cancer, and the growing number of immunological aspects encompassing sporadic colon cancer, the molecular and cellular links between infiltrating immune cells, regulation of the inflammation, and cancer development are not well understood. Therefore, we aimed to review the current information available on the role of T_{regs} in the pathogenesis of IBD, colitis-associated colon cancer and sporadic colorectal cancer.

T_{REGS} IN IBD

Discrepancy in the distribution of T_{regs} in peripheral blood and mucosa

T_{regs} are able to suppress the expansion of effector T cells by interleukin (IL)-10 and transforming growth factor (TGF)-β secretion and cytotoxic T lymphocyte-associated antigen expression[11]. The frequency of circulating T_{regs} was shown to be lower in patients with relapsing than in remitting autoimmune diseases[12,13], therefore leading to the speculation that insufficient levels of T_{regs} in peripheral blood are critical in inducing a relapse of autoimmune disease.

Maul *et al*[14] reported that CD4+CD25+ cells were decreased in the peripheral blood of IBD patients. Moreover, Wang *et al*[3] found the percentage of CD4+Foxp3+ T_{regs} in peripheral blood to be significantly lower in IBD (both ulcerative colitis and Crohn's disease) patients compared with healthy controls. However, at the mucosal level, Maul *et al*[14] and Wang *et al*[3] found the frequency of T_{regs} higher in active and inactive IBD samples compared to healthy controls. Their results and the results of Holmén *et al*[15] indicated that the increased frequency of CD25+ T cells in inflamed mucosa might be associated with the decreased number of circulating CD4+CD25+ T cells. During active inflammation, circulating T_{regs} migrate into the lamina propria to maintain homeostasis. As direct cell-to-cell contact is critical for T_{regs} to function, a low number of T_{regs} may result in a disturbance in the inhibition of the inflammatory reaction[15,16]. The results of Wang *et al*[3] suggest that an insufficient number of T_{regs} in the peripheral blood may be associated with the recurrence of IBD.

While Wang *et al*[3] did not find any alteration in the frequency of mucosal CD4+Foxp3+ T_{regs} in ulcerative colitis and Crohn's disease, Hovhannisyan *et al*[17] showed that the inflammatory environment in the colonic mucosa of Crohn's disease patients (but not in that of ulcerative colitis patients) contributes to the generation of a distinct population of T_{regs} that are Foxp3+ and produce IL-17. These Foxp3+ IL-17-producing cells also produced large amounts of interferon (IFN)-γ, another effector cytokine. On the other hand, Kryczek *et al*[18] observed mucosal Foxp3+IL17+CD4+ T_{regs} in ulcerative colitis that induce proinflammatory cytokines and suppress effector T cell function, exhibiting their dual inflammatory and regulatory function.

The balance between these subpopulations of mucosal T_{reg} cells, which may be regulated by the inflammatory milieu, seems to be crucial in the pathogenesis of IBD.

Phenotypic plasticity of T_{regs} in IBDs

The IL-17-producing CD4+ T (Th17) cells constitute a subset of helper T cells that has been linked to the pathogenesis of IBD[19]. Mucosal Th17 cells are thought to protect the host from infection.

The development of Th17 cells and T_{regs} requires TGF-β which suggests that both cell types may be related

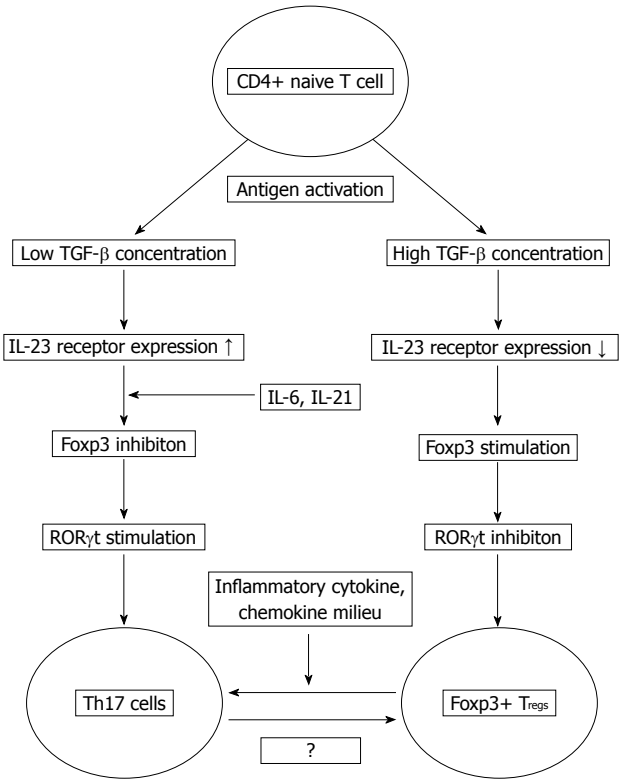


Figure 1 Phenotypic plasticity of T cells in inflammatory bowel disease. The mucosal concentration of transforming growth factor (TGF)-β influences the interleukin (IL)-23 receptor expression of antigen activated CD4+ T cells, which results imbalance in forkhead box P3 (Foxp3) and retinoic acid-related orphan receptor (RORγt) transcription factor activity. The Foxp3 dependent inhibition or stimulation of RORγt may regulate helper T cell (Th) 17 or Foxp3+ regulatory T cell (T_{reg}) differentiation. The inflammatory cytokine/chemokine milieu may also influence the phenotypic changes of T cells.

and may arise from the same precursor based on the distinct cytokine milieus[20-23]. TGF-β exposure of antigen-activated naive CD4+ T cells results in upregulation of Foxp3 and retinoic acid-related orphan receptor (RORγt), two transcription factors that regulate T_{reg} and Th17 cell differentiation[24]. Zhou *et al*[25] showed that at low concentrations, TGF-β synergizes with IL-6 and IL-21 to promote IL-23 receptor (IL23R) expression, favouring Th17 cell differentiation, while in high concentrations it represses IL23R expression and favours Foxp3+ T_{regs} differentiation. *In vitro*, TGF-β-induced Foxp3 inhibits RORγt function, at least in part through their interaction. Consequently, lamina propria Foxp3+RORγt+T cells produce less IL-17 than those that express RORγt alone. IL-6, IL-21 and IL-23 inhibit Foxp3-mediated inhibition of RORγt, therefore promoting Th17 cell differentiation. Based on these data it seems, that the decision of antigen-stimulated cells to differentiate into either Th17 or T_{reg} cell depends on the cytokine-regulated balance of Foxp3 and RORγt[25].

Hovhannisyan *et al*[17] identified such a Foxp3+ IL-17-producing CD4+ T_{reg} cell population in the lamina propria of Crohn's disease patients that shared phenotypic characteristics of both Th17 and T_{reg} cells, and showed potent suppressor activity *in vitro*. Their analysis of the TCR repertoire revealed a similar TCR β-chain variable region usage by Foxp3+IL-17+ and Foxp3+CD4+ T cells, suggesting that Foxp3+ IL-17-producing cells may come from Foxp3+ T_{regs} when exposed to unique inflammatory signals present in Crohn's disease (Figure 1).

Increased mucosal TGF-β and IL-6 expression have been detected in Crohn's disease[26,27]. Similar results were not found in ulcerative colitis associated mucosa[26,27]. These cytokines are supposed to promote the conversion of T_{regs} into effector T cells, while retaining their suppressive properties[17,20]. Despite the presence of cytokines involved in Th17 and T_{reg} cell differentiation, the generation of Foxp3+IL-17+ T_{regs} from normal lamina proprial or peripheral blood CD4+ T cells failed[17]. Therefore it is speculated that additional, presumably local, factors are also required for the induction of these cells *in vivo*. Kryczek *et al*[18] found that these inflammatory T_{regs} are induced from memory chemokine (CC motif) receptor 6+ T cells and T_{regs}. Furthermore, TGF-β, IL-2 and the presence of antigen presenting cells are also essential for their induction.

The better understanding of the origin and function of T_{regs}, and the interaction between effector and regulatory sites of mucosal immune cells in IBD may lead to new therapeutic possibilities.

T_{REGS} IN IBD-ASSOCIATED COLON CANCER

In mice, Faubion *et al*[28] showed that chronic inflammation arising from the bowel may induce thymic involution and T_{reg} cell suppression. This was suggested to lead to the enhancement of inflammation-mediated events that worsen IBD. Restoration of homeostasis through suppression of tumor necrosis factor (TNF)-α production and fortification of T_{regs} were proposed for human IBD patients[4]. These data on IBD may support to the concept that uncontrolled inflammation weakens the T_{reg}-mediated inhibition and increases the risk for inflammation-associated carcinogenesis.

In colitis-associated colon cancer, a high frequency of Foxp3+IL-17+CD4+ T_{regs} was found in the tumor, but not in the adjacent mucosa[18]. Though human tumor-infiltrating T_{regs} express limited effector cytokines[29], colitic Foxp3+IL-17+CD4+ T_{regs} express a moderate amount of IFN-γ, TNF-α and IL-2, and shares similar cytokine profiles to blood Foxp3+IL-17+ T_{regs}[18]. This cytokine profile reveals a phenotype for polyfunctional effector T cells similar to that observed in infectious diseases[30,31] and tumor-infiltrating Th17 cells in multiple human cancers[32]. These data indicate that Foxp3+IL-17+ T_{regs} express effector cytokines and are distinct from T_{regs} within the same inflammatory milieu.

Moreover, in suppressive assays, Foxp3+IL-17+ T cells from colitis-associated colon cancer were able to suppress T cell proliferation and IFN-γ production in a dose-dependent manner[18], which suggests that Foxp3+IL-17+ T cells belong to a functional T_{reg} cell population.

Kryczek *et al*[18] also found that T_{regs} induce the production of IL-1 and IL-6 in ulcerative colitis. IL-17 derived from Foxp3+IL-17+CD4+ T cells may also induce IL-6 expression[33,34]. As IL-1 and IL-6 play a crucial role in ulcerative colitis[35,36], it is possible that IL-17-producing Foxp3+CD4+ T cells together with Th17 cells may potentially contribute to the early tumorigenesis seen in chronic ulcerative colitis.

The role of pathogenic microbial infection in colitis-associated carcinogenesis is also known[4]. After pathogenic microbial challenges, a pro-inflammatory response mediated by effector T cells serves to eliminate pathogenic bacteria. Educated CD4+CD45RB^{low} T_{regs} then subsequently suppress inflammation, offering protection from immunological self-destruction. It was shown that IL-10-dependent T_{regs} protect from malignancy[37,38]. Deficiency in IL-10 increases susceptibility to IBD-associated colorectal cancer (CRC) with poor prognosis[39]. Mice lacking IL-10 were shown to be susceptible to colitis-associated CRC after *Helicobacter hepaticus* (*H. hepaticus*) infection, while their wild type counterparts infected with *H. hepaticus* had only minimal bowel alterations[37,38,40,41]. The lack of IL-10 leads to elevated levels of TNF-α, IL-6 and IL-17, therefore allowing chronic inflammation to persist[42]. After *H. hepaticus* infection, IL-10 insufficient CD4+ T cells preferentially recruit to a Th-17 phenotype in the case of a pro-inflammatory challenge[4-45]. It was also shown in mice that bacteria-triggered inflammation mobilizes neutrophils bearing TGF-β1 and IL-6, which may inhibit T_{regs} and promote Th-17 response, a phenomenon that has been linked to carcinogenesis[40]. Based on these results, one may conclude that colonic bacterial infections supposed to trigger protective IL-10-dependent T_{regs}, while, under hygienic conditions, individuals with insufficiently educated T_{regs} are predisposed to sustained inflammatory reactions and may subsequently developed cancers[4].

Acting downstream of TGF-β1 signaling, Runx proteins are the interacting and functional partners of R-Smad proteins[46]. Among Runx proteins, Runx3 is involved in the differentiation of immune cells[47], and acts as a homeostasis maintainer and tumor suppressor in the gastrointestinal epithelium[48,49]. In mice, Sugai *et al*[47] found that the loss of Runx3 in T cells resulted in suppression of T_{reg} cell function which lead to the development of colitis and colonic tumors (associated with luminal microorganisms) in Runx3$^{-/-}$ animals.

Further studies are needed to reveal the interaction between the commensal intestinal bacterial flora and T_{regs} for the better understanding of colitis-associated colorectal carcinogenesis.

T_{REGS} IN SPORADIC COLORECTAL CANCER

Presence of T_{regs} and disease outcome

Cancer cells have evolved to develop strategies of immunoevasion to escape control by the immune system[50]. There are quite numerous possible immunoevasion mechanisms that appear to affect diverse steps in cancer-specific lymphocyte priming, activation, and effector function. These mechanisms may also operate simultaneously[51,52]. Evidence is accumulating that CD4+CD25+Foxp3+ T_{regs} are crucially involved in cancer immunobiology[10]. The number of Foxp3+ T_{regs} was found to be significantly increased in the peripheral blood, lymph nodes and surrounding tumors of patients with colorectal cancer compared to that of healthy controls[20,53]. Several data show that increased frequencies of T_{regs} prognosticate a worse outcome for colon cancer patients, as this may reflect the inability of the immune system to adequately respond to cancer[54-56]. Moreover, studies aimed at reducing T_{reg} cell frequencies in experimental models have resulted in successful control of cancer[57].

On the contrary, others showed that an increased intratumoral Foxp3+ T_{reg} cell number correlates with a favourable clinical outcome[58]. This can be the result of mast cell-T_{reg} cell interaction, which induces T_{regs} to switch function and escalate inflammation in CRC without losing T-cell-suppressive properties in an IL6 and IL17 independent manner[59].

Szczepanik *et al*[60] found that the absolute number of T_{regs} in the peripheral blood of gastric cancer patients, especially in those with lymph node metastases was significantly decreased in comparison to that of healthy controls. This phenomenon was not observed in CRC patients. Their results suggest that the population of T_{regs} in peripheral blood does not simply mimic intratumoral T_{regs}.

Based on this data, one can conclude that when inflammatory cells that promote tumor progression dominate the immune response, T_{regs} may be beneficial in suppressing carcinogenesis. However, when T cells dominate the immune response, T_{regs} may promote disease progression by suppressing their anti-tumoral immunological effects. The discrepancy between the peripheral blood and local, intratumoral number phenotype of T_{regs} in sporadic CRC may have an effect on this theory, and should be further investigated.

Contribution of T_{regs} to carcinogenesis and cancer cell survival

The colonic adenoma-dysplasia-carcinoma sequence is associated less clearly with inflammation then it is with colitis-associated colon cancer. C57BL/6 mice heterozygous for a mutation in the *Apc* gene (Apc$^{Min/+}$) are genetically at risk for intestinal polyps and mimic early stages of sporadic CRC in humans[61]. It was observed, that Apc$^{Min/+}$ mice have higher serum and intestinal levels of TNF-α, IL-6 and IL-17 than matched wild type animals, which may indicate a subclinical inflammatory condition[4]. Elevated inflammatory cytokine levels were found in human colorectal cancer as well[62].

Using T_{reg} cell titration assay it was also shown, that T_{regs} from *H. hepaticus* infected donor Apc$^{Min/+}$ mice con-

sistently provided complete protection from cancer and more effectively suppressed TNF-α, IL-6 and IL-17 cytokine levels in tumor-prone tissues than T_{regs} from uninfected donor mice[4,63]. It is thought that the protection from cancer is attributable to T_{reg} cell functions, and is more dependent upon the former conditions of T_{regs} than that of the recipient mice.

In humans, Ma *et al*[64] revealed that CD4+CD25+Foxp3+IL-17+ T cells in CRC tissue express TGF-β and IL-10, the functional molecules of T_{regs}. This subset of T cells suppresses anti-tumoral peripheral CD8+ T cells in a tumor antigen-specific manner, therefore contributing to cancer development. If the effector CD8+ T cells could disengage from the suppression of CD4+CD25+Foxp3+IL-17+ T_{regs}, they might again proliferate and fight against tumor development.

It has been shown that chemokine (CC motif) ligand (CCL) 5 is highly expressed in colon tumor tissues from early-stage CRC compared with paired normal tissues[65]. CCL5 promotes tumor growth and metastasis by inducing tumor cell proliferation, angiogenesis, and expression of matrix metalloproteinases[66,67]. It can also diminish anti-tumoral immune responses by increasing the presence of tumor-infiltrating macrophages and T_{regs}[67]. Chang *et al*[68] have recently found that CCL5 expression could not only promote migration of T_{regs} to tumors, but also enhance their killing ability on CD8+ T cells. Furthermore, this enhanced killing ability was associated with an increase in production of TGF-β by T_{regs}. Based on these results, it seems that the production of CCL5 by cancer cells is a part of CRC's immunoevasion repertoire.

Further studies may help us to better understand the interaction between tumor cells and T_{regs} and to identify an unrevealed mechanism by which tumor cells induce immune tolerance in CRC.

CONCLUSION

Targeting T_{regs} may offer an important therapeutic strategy as an adjunct to treatment of patients.

A better understanding of the microenvironmental cues that influence T_{reg} cell commitment toward a given lineage and their balance between regulation and inflammation may result in new therapeutic targets in IBD and colitis-associated cancer.

T_{regs} and IL-10 producing Tr1 cells have the potential to prevent or cure colitis in graft-versus-host disease, a finding supported by a favourable safety profile was demonstrated in phase I clinical trials[69]. Although it must be confirmed in further clinical trials, these results emphasize that T_{regs} may also be promising tools for therapeutic applications in IBD[70]. Changing the distribution of peripheral and mucosal T_{regs} may also have therapeutic potential in colonic inflammations.

We presently lack sufficient data to determine the clinical effect of an immunotherapy targeting T_{regs} in patients with sporadic CRC. The unexpected finding[58] that tumor-infiltrating Foxp3+ T cells could be associated with a favourable prognosis in CRC, suggests the potential in targeting these cells in immune-based anti-cancer therapies. On the other hand, eliminating CD4+CD25+Foxp3+IL-17+ T_{regs} may help the effector CD8+ T cells to fight against cancer cells. Therefore this subset of T cells may offer a novel therapeutic target in the treatment of CRC[64]. In conclusion, these conflicting results should be appreciated with some discretion, as the mechanisms linking tumor-infiltrating Foxp3+ T_{reg} cell density with a favourable outcome remain equivocal.

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ORIGINAL ARTICLE

Incidence of port-site metastasis after undergoing robotic surgery for biliary malignancies

Quan-Da Liu, Jun-Zhou Chen, Xiao-Ya Xu, Tao Zhang, Ning-Xin Zhou

Quan-Da Liu, Jun-Zhou Chen, Xiao-Ya Xu, Tao Zhang, Ning-Xin Zhou, Department of Hepatobiliary Surgery, Institute of Hepatobiliary Gastrointestinal Disease, PLA Second Artillery General Hospital, Beijing100088, China
Author contributions: Liu QD, Chen JZ and Zhou NX designed the research; Liu QD, Chen JZ, Xu XY, Zhang T and Zhou NX performed the research work; Liu QD and Zhou NX wrote the paper.
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Correspondence to: Ning-Xin Zhou, MD, Department of Hepatobiliary Surgery, Institute of Hepatobiliary Gastrointestinal Disease, PLA Second Artillery General Hospital, West-city District, Beijing100088, China. zhounx301@163.com
Telephone: +86-10-66343089 Fax: +86-10-66343072
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Abstract

AIM: To investigate the incidence of clinically detected port-site metastasis (PSM) in patients who underwent robotic surgery for biliary malignancies.

METHODS: Using a prospective database, the patients undergoing fully robotic surgery for biliary malignancies between January 2009 and January 2011 were included. Records of patients with confirmed malignancy were reviewed for clinicopathological data and information about PSM.

RESULTS: Sixty-four patients with biliary tract cancers underwent robotic surgery, and sixty patients met the inclusion criteria. The median age was 67 year (range: 40-85 year). During a median 15-mo follow-up period, two female patients were detected solitary PSM after robotic surgery. The incidence of PSM was 3.3%. Patient 1 underwent robotic anatomatic left hemihepatectomy and extraction of biliary tumor thrombi for an Klatskin tumor. She had a subcutaneous mass located at the right lateral abdominal wall near a trocar scar. Patient 2 underwent robotic pancreaticoduodenectomy for distal biliary cancer. She had two metachronous subcutaneous mass situated at the right lateral abdominal wall under a same trocar scar at 7 and 26 mo. The pathology of the excised PSM masses confirmed metastatic biliary adenocarcinoma.

CONCLUSION: The incidence of PSMs after robotic surgery for biliary malignancies is relatively low, and biliary cancer can be an indication of robotic surgery.

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Key words: Robotic surgery; Trocar; Port-site metastasis; Recurrence; Biliary tract cancer

Peer reviewers: De Aretxabala Xabier, Professor of Surgery, Universidad de Chile, Santos Dumont 999, Santiago 8380000, Chile; Juli Busquets, MD, PhD, Department of Surgery, Hospital Universitari de Bellvitge, C/Feixa Llarga, S/N, 08907 Barcelona, Spain

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INTRODUCTION

Cholangiocarcinoma (CCA) is an epithelial malignant neoplasm arising from the bile ducts with features of cholangiocyte differentiation. CCA is the second most common primary hepatobiliary malignant cancer after hepatocellular carcinoma, and epidemiologic studies suggest its incidence is increasing worldwidely[1]. Advanced unresected CCA has an extremely dismal prognosis, with a median survival of < 24 mo; meanwhile, five-

year survival rates after R0 resection for hilar CCA are 11% to 41% and for distal CCA are 27% to 37%[2]. The treatment of CCA is challenging, and surgical therapy is the only therapeutic option with a chance of cure. Owing to its unique patterns of invasion and metastasis, the disease recurrence rate was very high (> 50%) after R0 resection, either locoregional recurrence or distant metastases[3,4]. Recently, cutaneous or subcutaneous metastasis from CCA and gallbladder cancer was also noted[5-7], especially port-site metastasis (PSM) after laparoscopic surgery[8-14].

Since Mouret performed the first laparoscopic cholecystectomy in 1987, this technique has rapidly become the gold standard for the management of cholelithiasis. In the past decade, laparoscopic and robotic techniques have quickly expanded to the indication of various malignancies[15-17]. The advantages of a minimally invasive surgery are well established, such as shorter convalescence and decreased analgesic requirements, along with better cosmetic results. Laparoscopic oncology continues to be confirmed with favorable outcomes[18,19], however, reports of PSMs, a phenomenon of tumor seeding or implantation at the port-site of entry of the laparoscopic trocars, remain an area of concern[8-14,20-26]. There are very limited reports on the risk of PSMs occurring from robotic surgery for malignancies[27,28].

The goal of our study was to determine the incidence of PSMs in patients undergoing robotic surgery for biliary malignancies, which can somewhat extrapolate whether robotic surgery is indicated for advanced biliary cancers or not.

MATERIALS AND METHODS

This study was conducted with the approval of the institutional review board of PLA Second Artillery General Hospital. We maintain a prospective database of all patients undergoing robotic procedures performed by the Department of Hepatobiliary Surgery, and we reviewed all cases performed between January 2009 and January 2011. All patients with a confirmed biliary malignancy who underwent robotic surgery were included. Patients with benign disease, those who had died postoperatively or lost postoperative following-up, and those whose procedure converted to open or laparotomy were excluded. Data abstracted included the patient's age at diagnosis, site of biliary tumor, primary histological diagnosis, American Joint Committee on Cancer (AJCC) stage, surgical procedures, intraoperative findings, presence of ascites, information regarding adjuvant radiochemotherapy after initial surgery, detection of PSM, time to the development of PSM, findings at the time of the diagnosis of PSM, and the overall survival for patients who developed PSMs. AJCC stage was assigned as delineated in the National Comprehensive Cancer Network Clinial Practice Guidelines on Hepatobliary Cancers (Version 2, 2012)[29]. The medical records were reviewed for all robotic procedures, including operative notes, radiology reports, pathology reports, and all involved progress notes. All patients included in this analysis had a regular every 3-mo follow-up physical examinations.

PSM was defined as subcutaneous tumor recurrence in the abdominal wall, near or within the scar tissue of the previous robotic-trocar site, which was detected either by clinical examination or by radiologic findings.

In all cases, the da Vinci Surgical System (Intuitive Surgical, Inc., Sunnyvale, CA) under a carbon dioxide pneumoperitoneum with the maximum intraabdominal pressure of 14 mmHg was used. On average, 5 or 6 trocars were placed during the procedure, and incision size ranged from 8 mm to 12 mm. One 12-mm trocar, usually infraumbilical, served as the camera port, and 3 robotic 8-mm trocars were placed 8-cm relative to each other. One or two additional 12-mm troars were used as assistant and choledochoscopic access. The entry into the abdominal cavity was achieved under direct visualization. The non-robotic trocar used in all cases was the Versaport trocar system (United States Surgical, North Heaven, Connecticut). The trocars were removed after the abdomen was deflated with the trocars in place. The 12-mm trocar incisions were closed in 2 layers at the fascia and the skin level, and the 8-mm trocar incisions were usually closed at the skin only with an attempt to close all layers. A lavage of the port sites with povidone-iodine solution was performed at the discretion of the surgeon.

Standard statistical analyses were used and performed using SPSS software, Version 17.0 (SPSS Inc., Chicago, IL). Descriptive statistics and proportions were used to report relevant demographic characteristics.

RESULTS

One hundred and ninety-four patients underwent robotic surgery procedures during the study period, including 64 cases with biliary cancers: intrahepatic biliary cystadenocarcinoma in 3, hilar CCA in 39, middle and distal biliary tract cancer in 11, and gallbladder cancer in 11. After exclusion of 2 patients with conversion to open procedures and 2 patients with postoperative death, we finally identified 60 patients who met inclusion criteria. Meanwhile, the 4 patients with open conversion or death were evaluated together when needed. The clinical characteristics of the patients are listed in Table 1.

All patients had no pre- and intra-operative evidence of distant intra-abdominal tumor metastasis. Eight severely jaundiced patients had an intraoperative finding of minor ascites. For retrieval of specimens, we usually applied surgical glove or ladle-like forcep to retrieve the freezing specimen or tumor thrombi intraoperatively, and use of retrieval bags for large specimens at the end of procedure. The postoperative primary histological diagnosis was biliary adenocarcinoma in 59 cases, biliary cystadenocarcinoma in 3, and miscellaneous in 2.

The tumor, node, metastasis stages of primary tumor type are outlined in Table 2. The robotic procedures

Table 1 Demographic and clinicopathological characteristics of the patients undergoing robotic surgery for biliary tract cancers

Characteristics	No. of patients (n = 64)
Age (yr)	
Median	67
Range	40-85
Gender	
Male	37
Female	27
Tumor locations	
Intra-hepatic biliary cancer	3
Hiliar biliary cancer	39 (1 conversion, 1 death)
Middle biliary cancer	1
Distal biliary cancer	10 (1 conversion, 1 death)
Gallbladder cancer	11
Conversion to open procedures	2
Presence of ascites	
Yes	8
No	56
Use of retrieval bags	
Yes	55
No	9
Postoperative adjuvant radiochemotherapy	
Yes	29
No	35
Port-site metastasis	2

Table 2 The American Joint Committee on Cancer stage of the 64 patients with biliary tract cancers

Stage	Hilar biliary cancer (n = 39)	Mid- and distal biliary cancer (n = 11)	Gallbladder cancer (n = 11)	Intrahepatic biliary cystadenocarcinoma (n = 3)
T1	1	1	0	0
T2	8	9	0	2
T3	14	1	4	0
T4	16	0	7	1

performed are listed in Table 3. Among 64 patients with biliary tract cancers, 2 patients converted to open procedures, with a rate of conversion of 3.1%. The postoperative morbidity and mortality are listed in Table 4.

The median follow-up period for all of the patients was 15 mo (range: 11-34 mo). PSMs were detected in 2 (3.3%) of the 60 patients, both of them were absent of intraoperative ascites. The interval between robotic surgery and detection of PSM was 27 mo in patient 1 and 7 mo in patient 2.

Patient 1 was a 51-year-old woman, who admitted with complaints of abdominal pain, jaundice and generalized pruritus in February 2009. A diagnosis of Klatskin tumor (Bismuth classification ⅢⅠb) was suspected. Robotic anatomatic left hemihepatectomy and extraction of biliary tumor thrombi were performed. During the procedures, the glove-fingers were used for retrieval of tumor thrombi specimens (Figure 1). The pathology revealed well-differentiated papillary adenocarcinoma and biliary tumor thrombi arising from the left biliary duct. In order to prevent local tumor recurrence, a prophylactic stereotactic body radiation therapy (SBRT) with total



Figure 1 During robotic procedure, the glove-fingers were used for retrieval of tumor thrombi specimens. 1, 2 and 3: The robotic arms, arm 1, 2 and 3.

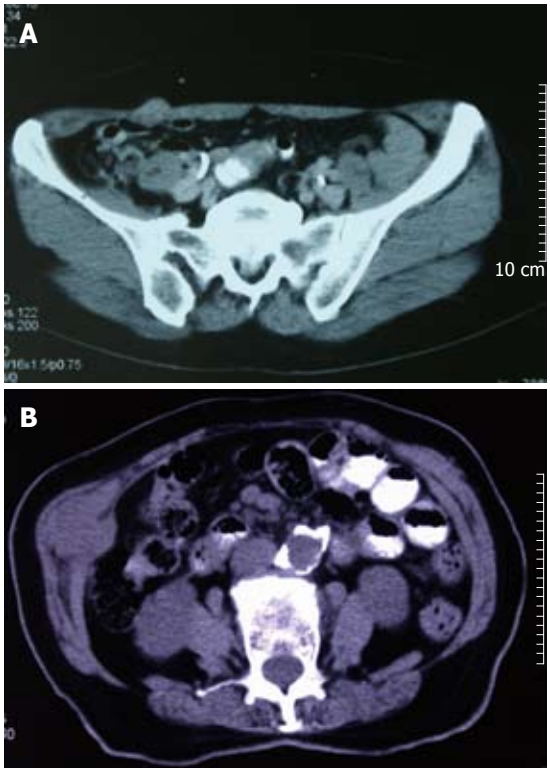


Figure 2 Computerized tomography scans of subcutaneous mass. A: A 2 cm × 1.5 cm subcutaneous mass at right lateral abdominal wall, which near a trocar scar on physical examination; B: A 4 cm × 3 cm × 3 cm subcutaneous mass at right lateral abdominal wall.

dose of 42 Gy (12 times) was given to the hepatic margin at 6th postoperative month.

In May, 2011, she was readmitted to our hospital for noting a subcutaneous mass at right lateral abdominal wall. Upon physical examination, a painless well-circumscribed, hard, subcutaneous mass near a trocar scar was palpated at the right lateral abdominal wall. Computed tomography (CT) scans confirmed a 2 cm × 1.5 cm subcutaneous mass (Figure 2A); no other obvious radiologic abnormalities were discovered. A subcutaneous tumor seeding was considered, and the mass was locally excised from the abdominal wall muscle (Figure 3A). Postoperative pathology confirmed metastatic biliary adenocarcinoma.

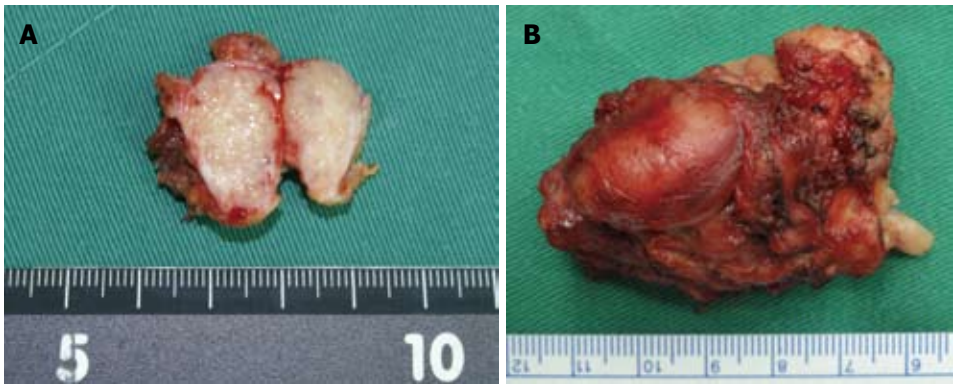


Figure 3 The subcutaneous mass was locally excised (A) and repeated excised locally (B) from abdominal wall muscle.

Table 3 The diagnosis and robotic surgical procedures of the 64 patients with biliary tract cancer

Tumor location	Surgical procedure	Number	Average time of procedures (min)
Intrahepatic biliary cystadenocarcinoma (n = 3)	Hepatic segmentectomy	3	220 (170-260)
Hilar biliary cancer (n = 39)	Anatomical left hemihepatectomy	3 (1 open conversion)	530 (410-650)
	Excision of tumor and GD-bridged biliary reconstruction	3	415 (390-460)
	Excision of tumor and Roux-en-Y hepaticojejunostomy	15	400 (350-510)
	Excision of tumor and biliary reconstruction	1	350
	Excision of tumor and external biliary drainage	7	210 (190-290)
	Excision of tumor and T-tube biliary drainage	10 (1 death)	230 (210-280)
Middle biliary cancer (n = 1)	Excision of tumor and Roux-en-Y hepaticojejunostomy	1	330
Distal biliary cancer (n = 10)	Whipple procedures	10 (1 conversion, 1 death)	720 (570-870)
Gallbladder cancer (n = 11)	Excision of GD tumor and Roux-en-Y hepaticojejunostomy	2	450 (410-490)
	Cholecystectomy	3	200 (170-300)
	Cholecystectomy and internal biliary drainage	1	220
	Cholecystectomy and T-tube biliary drainage	5	230 (210-280)

GD: Gallbladder.

Table 4 The postoperative morbidites and mortalities of the patients undergoing robotic surgery for biliary tract cancer (%)

Complications	No. of patients (n = 64)
Open conversion	2 (3.1)
Morbidity	9 (14.1)
Minor biliary leakage	3
Intra-abdominal hemorrhage	1
Pancreatic leakage	2
Pulmonary infection	2
Acute renal failure	1
Mortality	2 (3.1)
Pulmonary infection	1
Acute renal failure	1

In November 2011, about 33-mo after the robotic surgery, she was discovered multiple intraabdominal masses on CT scans, and was managed by SBRT.

Patient 2 was a 75-year-old woman with painless jaundice who was diagnosed as distal biliary tumor, and underwent robotic pylorus-preserving pancreaticoduodenectomy in July 2009. Postoperative pathology revealed poor-differentiated adenocarcinoma arising from the distal biliary duct. The preoperative serum CA199 level was 160 U/mL (normal range: 0-37 U/mL), then it dropped to normal range. An incisional hernia was occurred at infra-umbilical port site 2-mo after operation.

In February 2010, she had complained of a palpable painless subcutaneous mass of 3 cm × 2 cm size at right lateral abdominal wall under a robotic trocar scar, and which was removed by local excision. The pathology revealed seeding nodule of CCA. In September 2011, another 4 cm × 3 cm × 3 cm subcutaneous mass at the same site (Figure 2B) with obvious pain was noted, no other distal metastases were determined on CT scans. Meanwhile, the incisional hernia enlarged rapidly to a size of 13 cm × 13 cm; the serum CA199 level increased to 91.26 U/mL. Repeated local excision of subcutaneous mass (Figure 3B) and simultaneous incisional hernia repair with mesh implantation were performed. She recovered uneventfully. Postoperative pathology confirmed metastatic adenocarcinoma, and the serum CA199 level had returned to normal at that time.

DISCUSSION

Oncology-related laparoscopic PSMs were first reported

in 1978, Döbrönte *et al*[20] described a PSM occurring 2 wk after laparoscopy in a patient with malignant ascites. Since then, a number of PSMs cases after diagnostic or therapeutic laparoscopy have been reported, involving nearly all abdominopelvic malignancies[8-14,20-26]. However, given the sparse published data, and the few events of PSM, the precise incidence of PSM has still not been well defined. Ramirez *et al*[23] estimated that the overall incidence of PSMs after laparoscopic surgery was 1%-2%; and in general laparoscopic surgery the incidence of tumor seeding ranges from 0.8% to 21%[5,30]. Noticeably, the incidence of PSMs after laparoscopic cholecystectomy for incidental gallbladder cancer was as high as 14%-29% within the first 2 years after the initial operation[11-14].

Our study showed that the incidence of PSM in patients undergoing robotic surgery for the management of biliary malignancies is 3.3% (2/60). The 3.3% PSMs incidence in this study seems much lower than the reported incidence after laparoscopic surgery for incidental gallbladder cancer, and a little higher than that in general laparoscopic surgery[23]. The relatively low incidence of PSMs reveals that advanced biliary malignancies can be selected as an indication of robotic or laparoscopic techniques. To the best of our knowledge, this is the first study evaluating the incidence of PSMs after robotic procedures for biliary malignancies. Although there are rare reported cases of PSM after robotic procedures in malignancies[27,28], the risk of PSM between robotic and laparoscopic surgery would be not significantly different, because of procedures and requirements such as CO_2 pneumoperitoneum pressure (12-14 mmHg), the trocars placement are all much similar[28].

There are several postulated mechanisms for developing PSMs[23,30-34]. The most commonly discussed hypotheses include direct wound contamination and implantation, the biological aggressiveness of the primary tumor, haematogenous spread, effects of pneumoperitoneum that including the type of insufflating gas, chimney effect, aerosolization, surgical technique and the local immune response. Likewise the occurrence of PSMs is secondary to the above-mentioned multiple factors. Accordingly, several precautions were adopted with attempt to minimize the risk of PSMs[23,32-38].

The incidence of PSMs is much higher after laparoscopic surgery for incidental gallbladder carcinoma, which is mostly caused by tumor cells contamination due to gallbladder perforation during procedure and retrieval[8-14,39]. In this study, most of our patients had advanced biliary tract cancers. Their bile potentially contains tumor cells exfoliated from the primary tumor; bile ducts mobilization and dissection will probably detach more tumor cells into the bile; the robotic or laparoscopic instruments are inevitably contaminated by the spilled bile and exfoliated tumor cells. These possible scenarios resulted in consequent peritoneal carcinomatosis and PSM, especially in those with tumor thrombus, or undergoing R1 and R2 resection[5-7,40]. For patient 1, the procedures of tumor thrombi exposure and retrieval probably increase the risk of direct wound contamination of tumor cells, during specimen retrieval and instrument transfers.

This study raises a question regarding the indication of robotic or laparoscopic surgery for advanced biliary tract cancers. The initial result of this study justifies robotic surgery for biliary cancer on low incidence of PSMs. However, while either based on the surgical approaches, being robotic, laparoscopic or open, it is less important than the adherence to the principles of oncological surgery. All the procedures of violation of the primary tumor boundaries or damage of tumor-bearing lymph nodes, may promote tumor cell dissemination[41]. The conditions such as a presence of tumor thrombus and extensive intraabdominal metastases, may be a contraindication of robotic surgery for biliary tract cancer, because direct tumor manipulation infringes the oncological principles[41]. To prevent the trocar-site seeding in the case of biliary malignancy, we now routinely extract the specimen intact with the use of an impermeable laparoscopic retrieval bag[39]. Furthermore, we began to perform hyperthermal intraperitoneal chemotherapy at the end of robotic surgery to annihilate potential intraabdominal disseminated tumor cells[42].

In conclusion, the incidence of PSMs after robotic surgery for biliary cancer is relatively low, and biliary malignancies can be selected as an indication of robotic surgery. Although robotic surgery seemed more elaborate than laparoscopic surgery in managing biliary cancer, we should be aware of the risk of PSMs when dealing with malignancies. Emphasis on adhering to strict oncological surgical principles is the best method of prevention. Further studies are mandatory to better determine the role of robotic biliary surgery on the long-term oncological outcomes.

COMMENTS

Background

In the past decade, laparoscopic and robotic techniques have rapidly expanded to the indication of various malignancies. However, reports of port-site metastases (PSMs), a phenomenon of tumor seeding or implantation at the port-site of entry of the laparoscopic trocars, remain an area of concern. It has presumed that gallbladder cancer is a contraindication of laparoscopic technique owing to extremely high incidence of PSMs. This study estimates the incidence of clinically detected PSM in patients undergoing robotic surgery for biliary malignancies, in order to investigate the indication of biliary malignancies for robotic surgery.

Research frontiers

The laparoscopic technique has been little used for biliary malignancies, because the incidence of PSMs after laparoscopic cholecystectomy for incidental gallbladder cancer was as high as 14%-29% within the first 2 years after the initial operation. However, there are very limited reports on the risk of PSMs occurring from robotic surgery for malignancies, especially with no data of PSM incidence after robotic biliary surgery.

Innovations and breakthroughs

This is the largest number of case reports from a single-institution worldwidely. Total to sixty-four patients with biliary tract cancers underwent robotic surgery, and sixty patients met the inclusion criteria. During a median 15-mo follow-up period, two female patients were detected solitary PSM after robotic surgery. The incidence of PSM was 3.3%. The relatively low incidence of PSMs after

robotic surgery for biliary malignancies would arouse the interest of reappraisal the indication of laparoscopic or robotic procedures for biliary cancers.

Applications

Robotic surgery for biliary malignancies can be selected as an indication in selected patients with early or advanced biliary cancers, such as gallbladder cancer, Klatskin tumor and distal biliary cancer. Meanwhile, an emphasis on adhering to strict oncological surgical principles is the best method of prevention of PSMs, and further studies are mandatory to better determine the role of robotic biliary surgery on the long-term oncological outcomes.

Terminology

PSM is a phenomenon of tumor seeding or implantation at the port-site of entry of the laparoscopic trocars after laparoscopic or robotic surgery for malignancies. The high incidence would raise a question on the oncologic safety of laparoscopic or robotic procedures for malignancies.

Peer review

This is an interesting report of the author's experience in the management of patients with biliary malignancies by using robot.

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ORIGINAL ARTICLE

Traditional Chinese surgical treatment for anal fistulae with secondary tracks and abscess

Chen Wang, Jin-Gen Lu, Yong-Qing Cao, Yi-Bo Yao, Xiu-Tian Guo, Hao-Qiang Yin

Chen Wang, Jin-Gen Lu, Yong-Qing Cao, Yi-Bo Yao, Xiu-Tian Guo, Department of Anorectal Surgery, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China
Hao-Qiang Yin, Department of Imaging, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China
Author contributions: Wang C and Yao YB performed the majority of research and wrote the manuscript; Cao YQ and Guo XT coordinated and collected clinical materials; Yin HQ performed all ultrasound examinations; Lu JG designed this study and revised the manuscript; all authors read and approved the final version for submission.
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Correspondence to: Dr. Jin-Gen Lu, Department of Anorectal Surgery, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, No. 725 South Wanping Road, Shanghai 200032, China. wangchen_longhua@yahoo.com.cn
Telephone: +86-21-64385700 Fax: +86-21-64698179
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Abstract

AIM: To evaluate the efficacy and safety of traditional Chinese surgical treatment for anal fistulae with secondary tracks and abscess.

METHODS: Sixty patients with intersphincteric or transsphincteric anal fistulas with secondary tracks and abscess were randomly divided into study group [suture dragging combined with pad compression (SDPC)] and control group [fistulotomy (FSLT)]. In the SDPC group, the internal opening was excised and incisions at external openings were made for drainage. Silk sutures were put through every two incisions and knotted in loose state. The suture dragging process started from the first day after surgery and the pad compression process started when all sutures were removed as wound tissue became fresh and without discharge. In the FSLT group, the internal opening and all tracts were laid open and cleaned by normal saline postoperatively till all wounds healed. The time of healing, postoperative pain score (visual analogue scale), recurrence rate, patient satisfaction, incontinence evaluation and anorectal manometry before and after the treatment were examined.

RESULTS: There were no significant differences between the two groups regarding age, gender and fistulae type. The time of healing was significantly shorter (24.33 d in SDPC *vs* 31.57 d in FSLT, $P < 0.01$) and the patient satisfaction score at 1 mo postoperative follow-up was significantly higher in the SDPC group (4.07 in SDPC *vs* 3.37 in FSLT, $P < 0.05$). The mean maximal postoperative pain scores were 5.83 ± 2.5 in SDPC *vs* 6.37 ± 2.33 in FSLT and the recurrence rates were 3.33 in SDPC *vs* 0 in FSLT. None of the patients in the two groups experienced liquid and solid fecal incontinence and lifestyle alteration postoperatively. The Wexner score after treatment of intersphincter fistulae were 0.17 ± 0.41 in SDPC *vs* 0.40 ± 0.89 in FSLT and transsphincter fistulae were 0.13 ± 0.45 in SDPC *vs* 0.56 ± 1.35 in FSLT. The maximal squeeze pressure and resting pressure declined after treatment in both groups. The maximal anal squeeze pressures after treatment were reduced (23.17 ± 3.73 Kpa in SDPC *vs* 22.74 ± 4.47 Kpa in FSLT) and so did the resting pressures (12.36 ± 2.15 Kpa in SDPC *vs* 11.71 ± 1.87 Kpa in FSLT), but there were neither significant differences between the two groups and nor significant differences before or after treatment.

CONCLUSION: Traditional Chinese surgical treatment SDPC for anal fistulae with secondary tracks and abscess is safe, effective and less invasive.

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Key words: Traditional Chinese surgical treatment; Su-

ture dragging; Pad compression; Anal fistulae; Secondary tracks and abscess

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INTRODUCTION

Anal fistulae are common in human beings. Surgery remains one of the most effective therapies. The main objective of surgery for anal fistulae is to eradicate the fistula tract while maintaining anal continence[1]. Fistulotomy (FSLT) and fistulectomy are safe and effective for simple and superficial anal fistulae. When a fistula has secondary tracts or abscess, laying open procedures may have some complications such as longer healing time, larger defects, and higher risk of incontinence[2,3]. In China, many hospitals use traditional Chinese surgical therapies to treat anal fistulae. To some extent, these therapies work well[4-7]. Suture dragging combined with pad compression (SDPC) is a combined dynamic treatment which was invented by Bo-Hua Gu and has been applied to anorectal disease by Jin-Gen Lu since the 1980s[8]. The aim of this study was to evaluate the efficacy and safety of this traditional Chinese surgical treatment SDPC for anal fistulae with secondary tracks and abscess and to compare it with the surgical treatment FSLT in modern medicine.

MATERIALS AND METHODS

Subjects

A total of 60 consecutive patients with intersphincteric or transsphincteric anal fistulae with secondary tracks and abscess were included in the study, who were treated between January 2008 and March 2009 in the department of anorectal surgery, Longhua Hospital affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, China. This study was approved by the local ethical committee and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Patients with chronic inflammatory bowel disease, dermatitis, diabetes, tumors and pregnant or lactating women were excluded. Informed consent was obtained from all participants after full explanation of the procedure. None of them were dropped out or lost to follow-up after the study.

Treatment

Preoperative evaluation: Patients were randomly assigned to either the SDPC group or the FSLT group by computer-generated random number. Each group has 30 patients. Clinical evaluation was based on both digital examinations and assessments with perianal and endoanal ultrasonography by an experienced sonographer. All patients were identified to have either intersphincteric or transsphincter anal fistulae with secondary tracts or abscesses. Incontinence evaluation (Wexner score, 0-20) and anorectal manometry (Medtronic Synectics, Sweden) were performed with an 8 channel water-perfused catheter before surgery. Maximal anal squeeze and anal resting pressures were recorded.

Postoperative pain was evaluated using a visual analogue scale (0-10), incontinence evaluation and anorectal manometry were performed when wounds were completely epithelized.

Operative technique: All procedures were carried out by one of two surgeons with significant experience in SDPC and FSLT. Surgeon was told of the intervention one day before surgery. Both groups were operated upon under spinal anaesthesia and in lithotomy or left lateral position. One dose of metronidazole (Shanghai Huazhong Pharmaceutical, Ltd., Shanghai, China) was used intravenously during the operation.

In the SDPC group, internal opening and secondary tracts or abscess were identified by a ball-shape head sliver fistula probe (Figures 1 and 2). The internal opening and inflammatory tissue around were excised with scissors. Mucosa and internal sphincter below the internal opening were laid open and the incision was extended to anal verge for drainage. All secondary tracts and abscesses of intersphincteric fistulae or transsphincteric fistulae were curetted carefully to remove epithelialized and necrotic tissues. Incisions at external openings were made about 1 cm × 0.5 cm for drainage (Figure 3). Then ten silk sutures (4-0, 25 cm length, Shanghai Jinhuan Medical, Ltd., Shanghai, China) were put through every two incisions and knotted in loose state (Figure 4). For complete drainage, one incision was done in the middle of curved (Figure 5) or longer than 5 cm fistulous tract (Figure 6).

In the FSLT group, the internal opening and all tracts were identified with the same ball-shape head sliver fistula probe. The internal opening, extended fistula tracts, abscess and external openings were laid open thoroughly with scissors and curetted carefully. All wounds were left open for drainage.

Postoperative care: Metronidazole was administered intravenously for three days postoperatively in both groups. In the SDPC group, dressings were changed by doctors twice a day at 8 am and 4 pm, respectively. The suture dragging process was started from the first day after surgery during dressing change. Wounds and fistulous track cavities were irrigated with normal saline (Figure 7). Part of the silk sutures inside the fistula tract were dragged

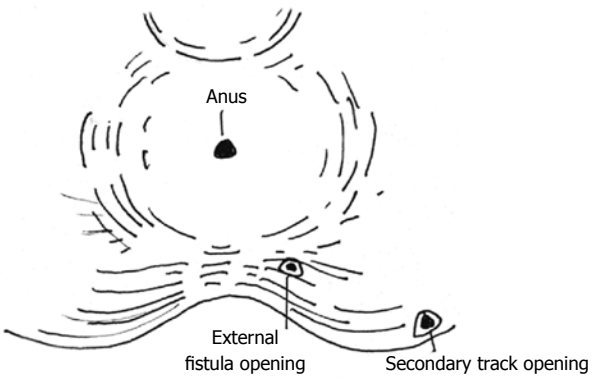


Figure 1 Low complex anal fistula.

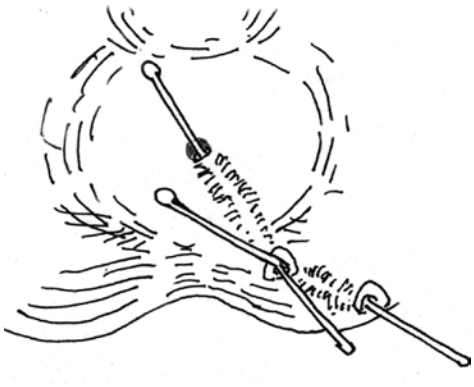


Figure 2 Internal opening and secondary tracts were found by a ball-shape head sliver fistula probe.

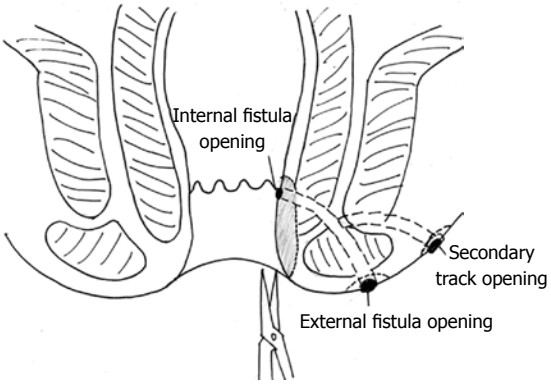


Figure 3 Mucosa and internal sphincter below the internal opening were laid open and a wound was made on anal verge for drainage. Secondary tracts or abscess were curetted.

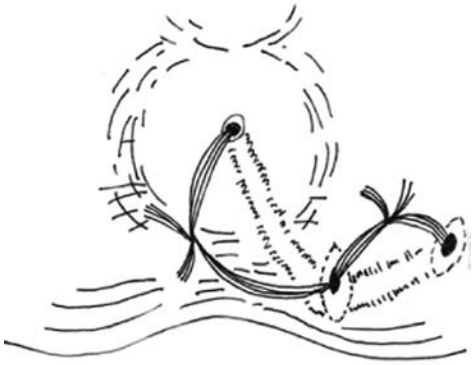
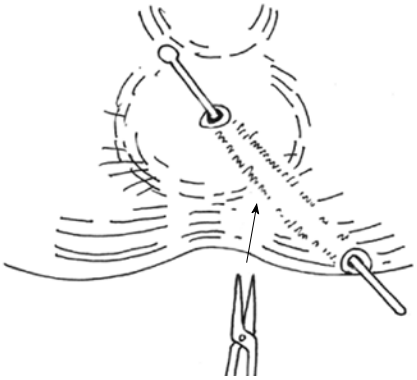


Figure 4 Silk sutures were put through every two incisions and knotted in loose state.



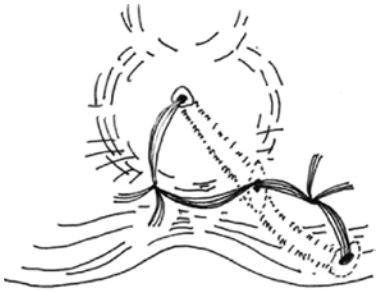
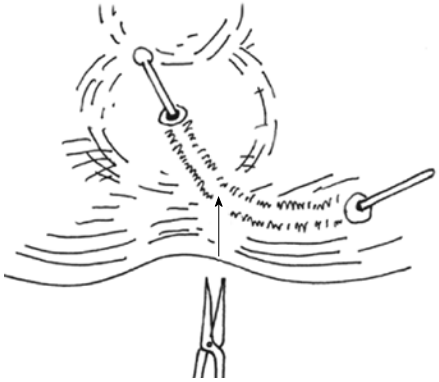


Figure 5 If fistulous track was longer than 5 cm, one incision was done in the middle of tract.



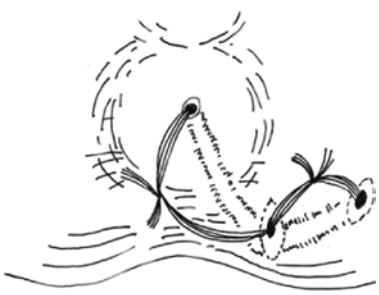


Figure 6 If fistulous track was curved, one incision was done at the top.

Table 1 Demographic characteristics and clinical outcomes of the patients

	SDPC (n = 30)	FIST (s = 30)	P value
Age (yr)	44.25 ± 14.31	41.17 ± 17.58	0.744
Gender (male/female)	22/8	19/11	0.405
Fistulae type			
Intersphincteric	6	5	0.739
Trans-sphincteric	24	25	
Secondary tracts	21	23	0.559
Secondary abscess	9	7	
Time of healing (d)	24.33 ± 4.44	31.57 ± 5.30	< 0.001
Postoperative pain scores	5.83 ± 2.50	6.37 ± 2.33	0.373
Oral analgesics (n)	10	14	0.292
Parenteral analgesics (n)	0	1	0.313
Recurrence rate (%)	3.33	0	0.313
Incontinence score			
Before treatment			
Intersphincteric	0.00 ± 0.00	0.20 ± 0.45	0.148
Trans-sphincteric	0.08 ± 0.28	0.12 ± 0.44	0.366
After treatment			
Intersphincteric	0.17 ± 0.41	0.40 ± 0.89	0.289
Trans-sphincteric	0.13 ± 0.45	0.56 ± 1.35	0.071
Manometry			
Maximal anal squeeze pressure (Kpa)			
Before treatment	24.73 ± 4.39	25.31 ± 3.89	0.590
After treatment	23.17 ± 3.73	23.74 ± 4.47	0.598
Resting pressure (Kpa)			
Before treatment	13.16 ± 2.23	12.66 ± 2.19	0.391
After treatment	12.36 ± 2.15	11.71 ± 1.87	0.212
Patient satisfaction	4.07 ± 0.94	3.37 ± 1.45	0.031

SDPC: Suture dragging combined with pad compression; FIST: Fistulotomy.

out and cleansed with normal saline, then turned back into the tract cavity. Usually the fistulous cavity became smaller at 10 d after surgery while the granulation tissue grew, then five sutures were cut and taken away. The residual sutures were removed one by one every 1-2 d afterwards depending on the space of the cavity. When all sutures were removed as wound tissue became fresh and without discharge, the pad compression process was initiated. One dose of lopramide hydrochloride (Xian-Janssen Pharmaceutical, Ltd., Xi'an, China) was given to the patients at the same time. A thick cotton pad was wrapped over the fistulous cavity and fixed with tape. Patients were asked to sit on a flat chair for 30 min, and relax for 10 min. The total time for pad compression was 6 h a day till the cavity healed.

In the FSLT group, dressings were changed by doctors twice a day at 8 am and 4 pm from the first day after surgery. Normal saline was used to clean the wounds until all wounds were completely epithelized. Patients in both groups were discharged when wounds healed. Success was defined by the absence of bleeding, pain, discharge, and subjective symptomatic relief.

Follow-up

Patients were followed up at outpatient clinic at 1 mo after the wounds were completely healed and by telephone at 12 mo after surgery. Patient's satisfaction was assessed in the outpatient clinic at the first follow-up using scores from -5 to +5 (with -5 being dissatisfied and +5 being very satisfied).

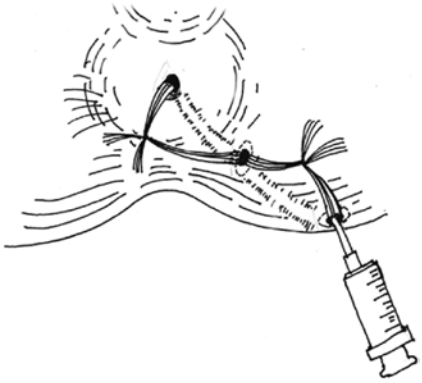


Figure 7 Wounds and fistulous track were irrigated with normal saline.

Statistical analysis

Statistical analysis was performed using SPSS for Windows Version 11.5 (SPSS Inc., Chicago, IL, United States). Descriptive statistics for demographic data were employed. Quantitative data were expressed as the mean ± SD, while χ^2 test was used for categorical data analysis. The two groups were compared with one-sided t test for analysis. $P < 0.05$ was considered significant.

RESULTS

The demographic data and outcomes in the two groups are shown in Table 1.

Time of healing

The mean time of healing in the two groups was 24.33 ± 4.44 d and 31.57 ± 5.30 d, respectively. It was significant shorter in the SDPC group than in the FSLT group.

Postoperative pain

The mean maximal postoperative pain scores were 5.83 ± 2.5 and 6.37 ± 2.33 for the SDPC group and the SDPC group, respectively. Ten (33.33%) patients in the SDPC group used oral analgesics and no patient required additional parenteral analgesics. Fifteen (46.67%) patients in the SDPC group used oral analgesics and one (3.33%) patient received parenteral analgesics due to severe pain.

Recurrence rate

One male patient in the SDPC group had swelling and discharged at week 3 after operation. He became asymptomatic after surgical drainage. All patients in the SDPC group were healed in one stage.

Incontinence evaluation

None of the patients had any incontinence before surgery. Two patients with transphincter fistula in the SDPC group and one with intersphincter and two with transsphincter fistula in the SDPC group used pads to prevent

underwear contamination. Postoperatively, no patient in the SDPC group experienced incontinence, but one intersphincter and two transsphincter fistulae patients used pads in the first two weeks after surgery. One female patient in the SDPC group with transsphincter fistula had transient gas incontinence from the third day after surgery. Symptoms of this patient disappeared 4 wk later without any treatment. Another one intersphincter and three transsphincter patients in the SDPC group wore pads in the first two weeks after surgery. The Wexner score after treatment of trans-sphincter fistulae was 0.13 ± 0.45 in the SDPC group and 0.56 ± 1.35 in the FSLT group (P = 0.071). None of the patients in this study experienced any problem with liquid and solid fecal incontinence and lifestyle alteration postoperatively.

Anorectal manometry

The maximal anal squeeze pressures before operation were 24.73 ± 4.39 Kpa in SDPC and 25.31 ± 3.89 Kpa in FSLT, and reduced to 23.17 ± 3.73 Kpa in SDPC and 22.74 ± 4.47 Kpa in FSLT after operation. The resting pressures before operation were 13.16 ± 2.23 Kpa in SDPC and 12.66 ± 2.19 Kpa in FSLT, and reduced to 12.36 ± 2.15 Kpa in SDPC and 11.71 ± 1.87 Kpa in FSLT after operation. Both of the maximal squeeze pressure and resting pressure declined, but there were neither significant differences between the two groups and nor significant differences before or after treatment.

Patient satisfaction

The mean satisfaction score at 1-mo postoperative follow-up for the SDPC group was 5 in ten patients, 4 in fifteen patients, 3 in three patients, 2 in one patient and 1 in one patient. It was 5 in six patients, 4 in ten patients, 3 in nine patients, 2 in two patients, 1 in one patient, 0 in one patient and -1 in one patient in the SDPC group. The mean satisfaction score was significant higher in the SDPC group (4.07 ± 0.94 in SDPC *vs* 3.37 ± 1.45 in FSLT).

DISCUSSION

The prevalence rate of anal fistulae is 8.6 cases per 100 000 populations[9]. Surgery is the most effective treatment for cryptoglandular fistula. For intersphincteric and low trans-sphincteric fistula, FSLT is a primary therapy[10]. Though complete FSLT is safe, it usually leads to larger wounds that take longer time to heal[11,12]. Recently, Atkin *et al*[13] reported that the laying open technique was still a good option for the management of complex anal fistulas. Ninety-six percent of their patients healed and the incidence rates of incontinence for flatus, soft stools and hard stools were low. But patients were unsatisfied with the postoperative complications of this laying open technique such as severe pain, longer healing time and unsightly scar[7]. More and more surgeons begin to seek less invasive therapies such as fibrin glue, sealant, anal fistula plug, endorectal mucosal advancement flap and ligation of intersphincteric fistula tract. Fibrin glue injection is technically easy, but results have been disappointing, with a success rate as low as 16%[14-16]. Similarly, the use of the anal fistula plug is a simple, low-risk but very expensive technique, and the reported success rates range between 29% and 87%[17-19]. Mucosal advancement flaps are technically difficult and associated recurrence rates vary from 2% to 54%[20-22]. Modified setons as cable-tie seton, silastic tube, silk, linen, braided silk, rubber band, and so forth were used to deal with complex or high anal fistula in recent years. However, incontinence still can not be avoided[23,24]. The latest conservative technique is ligation of intersphincteric fistula trac[25,26], but this procedure does not suit for fistula with abscess. Some novel techniques for treating complex fistula as video-assisted anal fistula treatment[27] and fistula laser closure[28], have been proposed. But these techniques need special equipments although the success rates were up to 80%. In this research, we found that the traditional Chinese surgical treatment SDPC can preserve the integrity of anal sphincter musculature involved by fistulous tract or abscess and maintain anal function with less postoperative pain and shorter healing time. This method has been used to treat fistulae and abscess diseases since the 1980s, which includes two processes. The "suture dragging" process refers to that the sutures which are put through the fistulous tracks or abscesses, are dragged by doctors postoperatively during dressing change. This process usually takes place at the first two weeks postoperatively. Suture dragging not only has the same functions of loose seton as mark and drainage, but also can stimulate granulation tissue to grow. The "pad compression" process means use thick pad to cover the wound and ask patients to sit on flat chair to accelerate the adhesion of wound cavity. Appropriate compression is good for tissue adhesion in wounds with deep cavity. In the last decade, some hospitals in China applied SDPC therapy to treat breast fistulae, acute mastitis, cutaneous abscess, anorectal horseshoe abscesses, necrotic fasciitis, pilonidal sinus disease and have obtained good results[29-32].

In this study, we used SDPC therapy to treat secondary tracts and abscess of intersphincteric and low transsphincteric fistulae. The SDPC therapy did not produce large wounds; the mean time of healing was reduced to 7 d compared with that of the FSLT. For the SDPC group, the postoperative pain mostly happened at dressing change. This was because some patients felt discomfort when doctor dragged sutures. But for the SDPC group, the postoperative pain was severe during bowel movement by stool evacuation. There was one male patient in the SDPC group complicated with edema and pain due to infection 3 wk after surgery during pad compression treatment. He suffered from diarrhea due to taking unhygienic food and not changing dressing regularly. After meticulous debridement and complete wound irrigation, he remained asymptomatic afterwards. One female patient who had gas incontinence

in the SDPC group had a complex trans-sphincteric fistula in which internal opening was at 6 o'clock and external openings at 6 o'clock and 7 o'clock, 1.5 cm and 2 cm from the anal verge respectively. The patient's symptom disappeared while wounds healed at 4 wk postoperatively. Though FSLT is safe in treating low trans-sphincteric fistula, it may damage part of external sphincter, especially for female patients with complex fistula. The Wexner score of trans-sphincteric fistula patients in the SDPC group was higher than in the SDPC group postoperatively. Maximal squeeze pressure and resting pressure after treatment reduced in both groups. This may be because that FSLT divided partial sphincter when managing the internal opening. But no patient experienced any problem concerning solid and liquid incontinence after treatment in both groups. Manometery confirmed that both therapies were safe. Most of the patients were highly satisfied with SDPC therapy for the advantages of less invasiveness, tiny scar, mild pain, shorter healing time and hospitalization.

Notably, the pattern of medical treatment in China is different from that of the Western countries. Doctors take care of patients' wounds postoperatively and patients can be discharged until their wounds are mostly epithelialized. On the contrary, in most developed countries, the procedures for most benign anorectal conditions are usually performed in outpatient settings, and patients can take care of their wounds themselves after hospital discharge. However, the treatment outcomes are quite good in this research, possibly due to this special pattern of treatment.

However, there are some limitations of this study. First, the sample size was small, and follow-up period was not long enough to assess the recurrence. SDPC therapy for complex trans-sphincteric fistulae is superior to that for intersphincteric fistulae. For intersphincteric fistulous tracks are usually short and laying open of this kind of fistulae is easy and safe. But for longer fistulous tracts and secondary abscesses, the advantages of this traditional Chinese surgical treatment are more apparent. In addition, the SDPC and FSLT are different techniques, it is impossible to be blinded to either surgeons or patients. In the future, we will conduct multi-center randomized controlled trials to compare SDPC with "cutting seton" or FSLT for high and low complex trans-sphincteric and supersphincteric anal fistulae.

As one of the traditional Chinese surgical therapies, the SDPC is successful in the treatment of perianal fistulae and abscesses disease. But the rationale of this treatment remains unclear. Why could dragging threads stimulate granulation tissue growth? When is the best time to remove sutures and start pad compression? What kind of appropriate pressure and duration can accelerate tissue adhesion without ischemia? Only when these questions have been clearly answered, can this traditional Chinese surgical sphincter-saving method be well accepted. More rigorous prospective randomized controlled trials with a larger sample size and longer follow-up are urgently needed.

In conclusion, traditional Chinese surgical treatment SDPC is safe, effective and less invasive. It can be used as an alternative to the treatment of complex anal fistulae.

COMMENTS

Background

Surgery is one of the most effective therapies for anal fistulae. More and more surgeons begin to seek less invasive therapies. However, classical procedures such as fistulotomy (FSLT) and fistulectomy, may produce complications such as longer healing time, larger defects, and higher risk of incontinence when dealing with fistulae with secondary tracts or abscess. In China, traditional Chinese surgical method suture dragging combined with pad compression (SDPC) has been used to treat anal fistulae and achieved good results.

Research frontiers

The postoperative complications of anal fistulae mostly include high risk of incontinence, severe pain, longer healing time and unsightly scar. Surgeons begin to seek less invasive therapies such as fibrin glue, sealant, anal fistula plug, endorectal mucosal advancement flap and ligation of intersphincteric fistula tract. The hotspot of anal fistula surgery is how to eradicate the fistula tract while maintaining anal continence and improving patient's quality of life.

Innovations and breakthroughs

There has been few studies about traditional Chinese surgical treatment for fistula diseases. This study reported that the traditional Chinese surgical method is effective and safe compared with the method used in modern medicine. The SDPC used in this study was less invasive with a higher patient's satisfaction.

Applications

The study results suggest that traditional Chinese surgical method SDPC is safe, effective and less invasive. It could be used as an alternative to the treatment of complex anal fistulae.

Terminology

Traditional Chinese surgical treatment SDPC includes two processes. The "suture dragging" refers to that the sutures which were put through the fistulous tracks or abscess during the operation, are dragged by doctor postoperatively during dressing change. The "pad compression" means use thick pad to cover the wound and ask patients to sit on flat chair to accelerate the adhesion of wound cavity. By this method, surgeons can preserve the integrity of anal sphincter musculature involved in fistulous tract or abscess, and maintain anal function with less postoperative pain and shorter healing time.

Peer review

The article presents an interesting novel technique to show the Western world another method of treatment. There was a significant shorter time to healing and higher patients' satisfaction in the study group, without difference in continence or complications. The described technique seems interesting, however it takes a greater deal of postoperative care compared with the traditional FSLT.

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ORIGINAL ARTICLE

Ethyl pyruvate protects against experimental acute-on-chronic liver failure in rats

Lu-Wen Wang, Li-Kun Wang, Hui Chen, Cheng Fan, Xun Li, Can-Ming He, Zuo-Jiong Gong

Lu-Wen Wang, Li-Kun Wang, Cheng Fan, Xun Li, Can-Ming He, Zuo-Jiong Gong, Department of Infectious Diseases, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China
Hui Chen, Institute of Infectious Diseases, Hubei Center for Disease Control and Prevention, Wuhan 430079, Hubei Province, China
Author contributions: Gong ZJ conceived and designed the research; Wang LW, Wang LK, Chen H, Fan C, Li X and He CM performed the research; Wang LW, Wang LK and Li X analyzed the data; Wang LW drafted the manuscript; Gong ZJ revised the manuscript; all authors have read and approved the final version to be published.
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Correspondence to: Zuo-Jiong Gong, MD, PhD, Professor, Department of Infectious Diseases, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuchang District, Wuhan 430060, Hubei Province, China. zjgong@163.com
Telephone: +86-27-88041911 Fax: +86-27-88042922
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Abstract

AIM: To investigate the protective effects of ethyl pyruvate (EP) on acute-on-chronic liver failure (ACLF) in rats.

METHODS: An ACLF model was established in rats, and animals were randomly divided into normal, model and EP treatment groups. The rats in EP treatment group received EP (40 mg/kg) at 3 h, 6 h, 12 h and 24 h after induction of ACLF. Serum endotoxin, high mobility group box-1 (HMGB1), alanine transaminase (ALT), tumor necrosis factor-α (TNF-α), interferon-α (IFN-γ), interleukin (IL)-10 and IL-18 levels, changes of liver histology and HMGB1 expressions in liver tissues were detected at 48 h after induction of ACLF. The effects of EP on the survival of ACLF rats were also observed.

RESULTS: Serum levels of endotoxin (0.394 ± 0.066 EU/mL *vs* 0.086 ± 0.017 EU/mL, $P < 0.001$), HMGB1 (35.42 ± 10.86 μg/L *vs* 2.14 ± 0.27 μg/L, $P < 0.001$), ALT (8415.87 ± 3567.54 IU/L *vs* 38.64 ± 8.82 IU/L, $P < 0.001$), TNF-α (190.77 ± 12.34 ng/L *vs* 124.40 ± 4.12 ng/L, $P < 0.001$), IFN-γ (715.38 ± 86.03 ng/L *vs* 398.66 ± 32.91 ng/L, $P < 0.001$), IL-10 (6.85 ± 0.64 ng/L *vs* 3.49 ± 0.24 ng/L, $P < 0.001$) and IL-18 (85.19 ± 3.49 ng/L *vs* 55.38 ± 1.25 ng/L, $P < 0.001$) were significantly increased, and liver tissues presented severe pathological injury in the model group compared with the normal group. However, EP administration significantly improved hepatic histopathology and reduced the serum levels of endotoxin (0.155 ± 0.045 EU/mL *vs* 0.394 ± 0.066 EU/mL, $P < 0.001$) and inflammatory cytokines (11.13 ± 2.58 μg/L *vs* 35.42 ± 10.86 μg/L for HMGB1, 3512.86 ± 972.67 IU/L *vs* 8415.87 ± 3567.54 IU/L for ALT, 128.55 ± 5.76 ng/L *vs* 190.77 ± 12.34 ng/L for TNF-α, 438.16 ± 38.10 ng/L *vs* 715.38 ± 86.03 ng/L for IFN-γ, 3.55 ± 0.36 ng/L *vs* 6.85 ± 0.64 ng/L for IL-10, and 60.35 ± 1.63 ng/L *vs* 85.19 ± 3.49 ng/L for IL-18, respectively, $P < 0.001$), and the levels of HMGB1 in liver tissues regardless of treatment time after induction of ACLF. EP treatment at the four time points prolonged the median survival time of ACLF rats (60 h) to 162 h, 120 h, 102 h and 78 h, respectively ($\chi^2 = 41.17$, $P < 0.0001$).

CONCLUSION: EP administration can protect against ACLF in rats, and is a potential and novel therapeutic agent for severe liver injury.

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Key words: Acute-on-chronic liver failure; Ethyl pyruvate; High mobility group box-1; Inflammatory cytokines; Histopathology; Survival time

Peer reviewers: Parimal Chowdhury, PhD, Department of Physiology and Biophysics, Faculty of Medicine, University of Arkansas for Medical Sciences, 4301 W Markham Street, Little Rock, AR 72205, United States; James Neuberger, Professor,

Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom

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INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a severe life-threatening condition with a high fatality, which still lacks effective treatment options[1]. Evidences have indicated that intestinal endotoxemia plays a significant role in the pathogenesis and progression of liver failure[2,3], which results from overgrowth of gram-negative bacteria in gut, and/or bacteria translocation into the peritoneal cavity due to the high permeability of intestinal wall[4,5].

Recent studies have shown that high mobility group box-1 (HMGB1) protein is involved in the progression of endotoxemia[6-8]. HMGB1 can be released into the extracellular milieu by activated macrophages, immunostimulated gut epithelial cells and necrotic or damaged cells in a delayed manner relative to the secretion of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1[9-11]. HMGB1 contributes to the pathogenesis of many diverse inflammatory disorders[12-14]. A growing number of studies have indicated that HMGB1 is a successful therapeutic target in numerous experimental models, such as ischemia/reperfusion[15], acute respiratory distress syndrome[16], rheumatoid arthritis[17], sepsis[18], and cancer[19]. Therefore, we proposed that HMGB1 as an important inflammatory mediator of endotoxemia should also play a significant role in the pathogenesis of liver failure[20].

Ethyl pyruvate (EP), a simple aliphatic ester derived from pyruvic acid, has been shown to improve survival and ameliorate organ dysfunction in a variety of animal models of critical illness, including acute endotoxemia[21], polymicrobial bacterial sepsis[22], postsurgical ileus[23], burn injury[24], acute pancreatitis[25], organ ischemia and reperfusion injury[26,27], and hemorrhagic shock[28]. As an effective anti-inflammatory agent, EP inhibits the activation of key inflammatory signaling pathways[29,30], and down-regulates the secretion of multiple inflammatory proteins, including IL-6, TNF-α and HMGB1[31-33]. These findings suggest that by reducing the levels of proinflammatory mediators, EP could also be a good candidate for treating or protecting against liver failure.

In the present study, we established an ACLF rat model, and investigated whether EP had protective effects on ACLF.

MATERIALS AND METHODS

Experimental animals

Female specific pathogen free Wistar rats, weighing 180-220 g, were purchased from the Experimental Animal Center of Wuhan University. All animals were acclimated to the laboratory for 5 d before experiment and were maintained in a light-controlled room (12-h light/dark cycle) at an ambient temperature of 25 ℃ with free access to water and standard chow. All animals were given humane care in accordance with the institutional guidelines.

Establishment of animal models

The ACLF rat models induced by human serum albumin (HSA), D-galactosamine (D-Gal) and lipopolysaccharide (LPS) were established as described elsewhere[34]. In brief, an immune liver fibrosis rat model was firstly induced by HSA (Octapharma GmbH, Austria)[35,36]. HSA was diluted to a concentration of 8 g/L with physiological saline and emulsified with an equal amount of incomplete Freund's adjuvant. The rats were injected subcutaneously at multipoints with 0.5 mL above solutions containing 4 mg HSA for a total of four times (a 14-d interval between the first and second time; a 10-d interval between the third and fourth time). Then, 4 mg HSA was injected into rat tail vein twice a week for 6 wk. Next, the rats with immune liver fibrosis were injected intraperitoneally with D-Gal (a purity of 98%, Sigma-Aldrich Co., United States) at a dose of 400 mg/kg combined with LPS (a purity of 99%, Sigma-Aldrich Co., United States) at a dose of 100 μg/kg to induce acute liver failure on the basis of chronic liver injury[37,38]. The normal rats were administrated with physiological saline (i.e., the vehicle solution of HSA, D-gal and LPS) at the same time points as did in the model rats, which served as a control.

Experimental design

Animals were randomly divided into normal group (n = 15), model group (n = 15) and EP treatment group (n = 60). The rats of EP treatment group were further divided into four subgroups (each subgroup n = 15), which were injected intravenously with Ringer's EP solution, containing Na^+ 130 mmol/L, K^+ 4 mmol/L, Ca^{2+} 2.7 mmol/L, Cl^- 139 mmol/L, and EP 28 mmol/L, pH = 7.02 (EP with a purity of 98% was purchased from Sigma-Aldrich Co., United States), at a dose of 40 mg/kg[39,40] at 3 h, 6 h, 12 h and 24 h. The time of combined administration of D-Gal and LPS was accepted as baseline (time point 0). EP was administrated subsequently at different time points. The rats of normal and model groups were injected intravenously with the same volume of Ringer's solution at the same time points. Five rats were sacrificed randomly for blood and hepatic tissue sampling at 48 h in each group. The remaining ten rats were observed for survival every 12 h for 7 d after induction of ACLF.

Blood sample examination

The serum levels of endotoxin were examined with Limulus Amebocyte Lysate (LAL, QCL-1000™, BioWhittaker, United States) according to the manufacturer's protocols. The serum levels of HMGB1, TNF-α, IFN-γ, IL-10 and IL-18 were measured using a rat HMGB1

enzyme linked immunosorbent assay (ELISA) Kit (Wuhan EIAab Science Co., Ltd, China) and commercial ELISA kits (Jingmei Biotech Co., Ltd, China) following the manufacturer's instructions, respectively. The alanine transaminase (ALT) level was determined by routine biochemical methods using a Hitachi Automatic Analyzer (Hitachi, Inc., Japan).

Histological examination

Liver samples were fixed in 10% buffered formalin for 24 h, embedded in paraffin, sliced into 5-μm thick sections and stained with hematoxylin and eosin (HE) to evaluate the pathological changes of the liver. The other portions of liver specimens were fixed in 2.5% glutaraldehyde, embedded in epoxy resin, sliced into ultrathin sections (60 nm in thickness) and stained with uranyl acetate and lead citrate to observe the ultrastructural changes of liver tissues under transmission electron microscope.

Real-time reverse transcription-polymerase chain reaction of HMGB1 mRNA

Total RNA was extracted from approximately 100 mg frozen liver tissues using TRIzol reagent (Invitrogen Co., United States). Two μg total RNA was reverse-transcribed into cDNA using a random primer hexamer (Promega Corporation, United States) and a MultiScribe™ Reverse Transcriptase (Applied Biosystems, United States). Gene expression was measured in real-time using primers and QuantiTect SYBR green polymerase chain reaction (PCR) Kit (QIAGEN Co., Ltd., China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers for rat HMGB1 were 5'-CGGATGCTTCTGTCAACTTCT-3' and 5' -AGTTTCTTCGCAACATCACCA-3'; for rat GAPDH, were 5'-TGCACCACCAACTGCTTA-3' and 5'-GGATGCAGGGATGATGTT-3'. Thermal cycling conditions included pre-incubation at 50 ℃ for 2 min, 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 30 s, 58 ℃ for 30 s and 72 ℃ for 45 s. LightCycler (Roche, Switzerland) was used to collect data automatically and analyze the value of threshold cycle (Ct). The fold change of HMGB1 mRNA expression in the model group or EP treatment group relative to the control group was detected using $2^{-\Delta\Delta Ct}$ method[41].

Western blot analysis of HMGB1 protein

In brief, 50 μg protein extracts from 100 mg liver tissue samples were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a Protran® nitrocellulose membrane (Schleicher and Schuell BioScience GmbH, Germany). The membrane was incubated with primary rabbit anti-rat HMGB1 antibodies (Boisynthesis Biotechnology Co., Ltd., China), and then with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Golden Bridge Biotechnology Co., Ltd., China), and finally detected by chemiluminescence using Enhanced NuGlo™ Chemiluminescent Substrate Kit (Alpha Diagnostic Intl. Inc., United States) followed by autoradiography and densitometric analysis. Membranes were also probed for β-actin as an additional loading control.

Immunofluorescence and confocal microscopy of HMGB1

Briefly, tissue sections were deparaffinized in xylene and rehydrated in a series of alcohol dilutions. The tissue sections underwent antigen retrieval using a commercial regent (Boster Biotechnology Co., Ltd., Wuhan, China), blocked with 10% normal goat serum in a humid chamber at 37 ℃ for 20 min to reduce non-specific binding, and then incubated with primary rabbit anti-rat HMGB1 polyclonal antibodies at 1:200 dilution at 4 ℃ overnight. The sections were then incubated away from light with a secondary fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Boster Biotechnology Co., Ltd., China) for 60 min at 37 ℃, counterstained with 2 μg/mL propidium iodide (Promoter Bio Co., Ltd., China) for 10 min, and observed under a Leika DM-IRE2 confocal microscope.

Statistical analysis

Results were expressed as mean ± SD. Differences among groups were assessed using unpaired Student *t* test and one-way analysis of variance. Animal survival data were evaluated using the Kaplan-Meier method and compared with the log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of EP on serum endotoxin, HMGB1, ALT and inflammatory cytokines

As shown in Figure 1, serum levels of endotoxin, HMGB1, ALT, TNF-α, IFN-γ, IL-10 and IL-18 were all significantly increased in rats of model group (0.394 ± 0.066 EU/mL, 35.42 ± 10.86 μg/L, 8415.87 ± 3567.54 IU/L, 190.77 ± 12.34 ng/L, 715.38 ± 86.03 ng/L, 6.85 ± 0.64 ng/L and 85.19 ± 3.49 ng/L, respectively) at 48 h after induction of ACLF compared with normal group (0.086 ± 0.017 EU/mL, 2.14 ± 0.27 μg/L, 38.64 ± 8.82 IU/L, 124.40 ± 4.12 ng/L, 398.66 ± 32.91 ng/L, 3.49 ± 0.24 ng/L and 55.38 ± 1.25 ng/L, $P < 0.01$). However, compared with model group, the serum levels of endotoxin, HMGB1, ALT, TNF-α, IFN-γ, IL-10 and IL-18 were all markedly lowered in EP treatment group at each time point, even at 24 h (0.327 ± 0.064 EU/mL, 15.73 ± 4.18 μg/L, 4863.78 ± 1864.13 IU/L, 143.24 ± 6.45 ng/L, 526.00 ± 28.68 ng/L, 4.96 ± 0.96 ng/L and 66.66 ± 5.59 ng/L, respectively, $P < 0.01$). There were significant differences in serum levels of endotoxin, HMGB1, TNF-α, IFN-γ, IL-10 and IL-18 in the EP treatment group at 24 h and at 3 h (0.155 ± 0.045 EU/mL, 11.13 ± 2.58 μg/L, 128.55 ± 5.76 ng/L, 438.16 ± 38.10 ng/L, 3.55 ± 0.36 ng/L, 60.35 ± 1.63 ng/L, $P < 0.05$).

Effect of EP on liver histology

HE staining showed a normal structure of hepatic lobule in the rat liver tissues of normal group. Neither de-

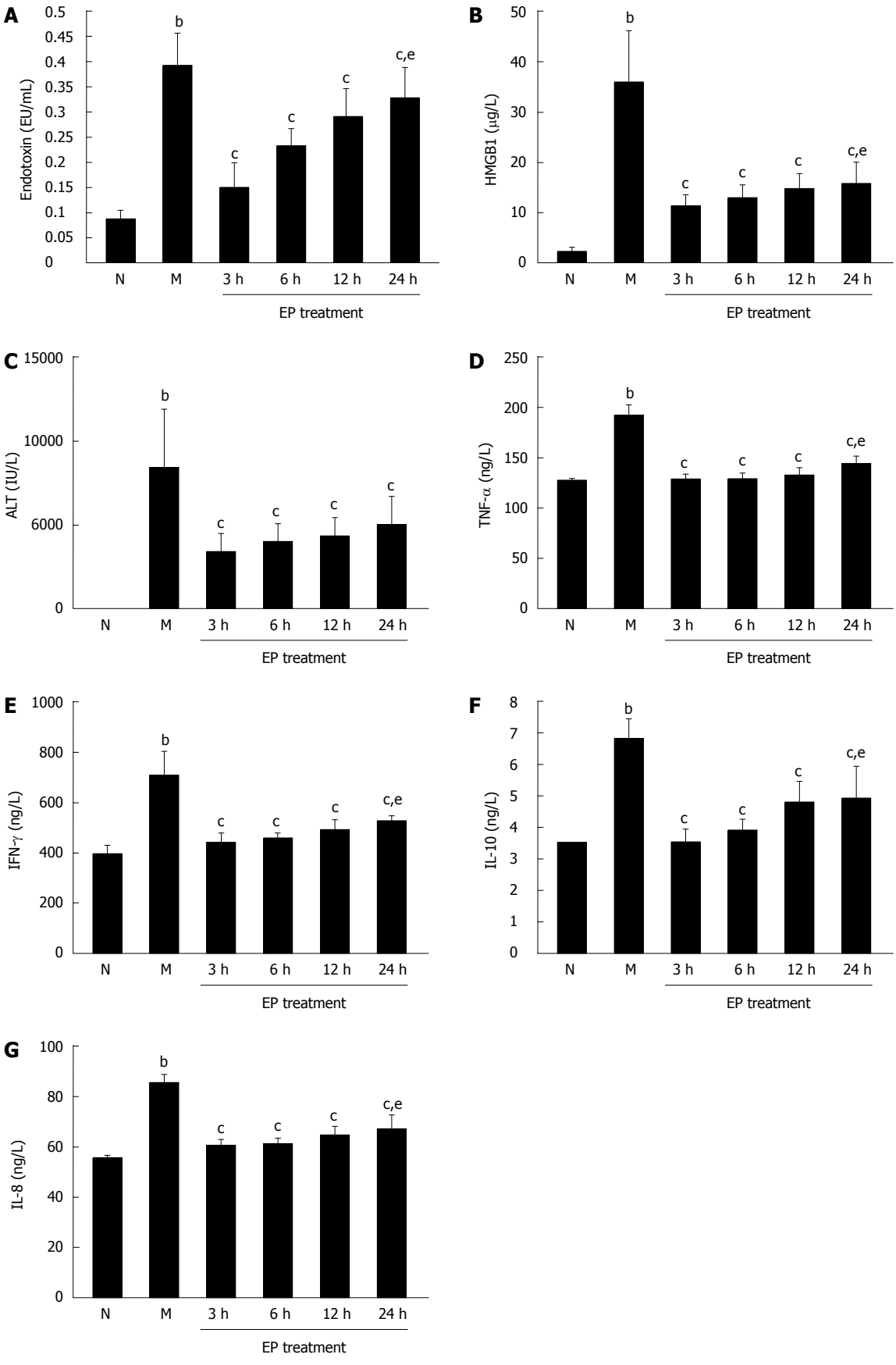


Figure 1 Effect of ethyl pyruvate on serum endotoxin, high mobility group box-1, hepatic enzyme and inflammatory cytokines in rat acute-on-chronic liver failure model. A: Endotoxin; B: High mobility group box-1 (HMGB1); C: Alanine transaminase (ALT); D: Tumor necrosis factor-α (TNF-α); E: Interferon-γ (IFN-γ); F: Interleukin (IL)-10; G: IL-18. $^{b}P < 0.01$ *vs* normal; $^{c}P < 0.05$ *vs* model; $^{e}P < 0.05$ *vs* ethyl pyruvate (EP) treatment at 3 h. Results are expressed as mean ± SD ($n = 5$). N: Normal group; M: Model group.

generation nor necrosis was presented. Hepatocytes were arrayed radiatively around the central vein (Figure 2A1). In the rat liver tissues of model group, the structure of hepatic lobules was in disorder with infiltration of many periportal inflammatory cells, hepatic plate was dissociated, and hepatocytes were swelling or with lytic necrosis. Massive or submassive necrosis of the regenerated nodules was revealed in some areas, while fibrosis septa were intact (Figure 2B1). However, liver histology was significantly improved in rats of EP treatment group as compared with the model group regardless of different time points of treatment. There was an obvious decrease of inflammatory cell infiltration, well-arranged hepatic cords, alleviation of hepatocyte swelling and less pyrenolysis (Figure 2C1, D1, E1 and F1). Under electronic microscope, the hepatocyte ultrastructure of rats in the normal group appeared normal (Figure 2A2), while in the model group, the glycogen granules decreased obviously in cytoplasm, endoplasmic reticulum was dilated and broken, mitochondrion was swollen, mitochondrial cristae were lysed, chromatin appeared vanishing, and even vacuolization occurred in the nuclei (Figure 2B2). Compared with the model group, hepatocyte ultrastructure was significantly improved in rats of EP treatment group, which showed that both endoplasmic reticulum dilation and mitochondrion swelling were alleviated obviously. Mitochondrial cristae and membrane remained clear, chromatin was symmetrical, and nucleoli and nuclear membrane were also legible (Figure 2C2, D2, E2 and F2).

Effect of EP on HMGB1 expression in liver tissues

The real-time reverse transcription-PCR showed that the expression levels of HMGB1 mRNA in the liver tissues of rats in the model group were significantly higher than in the normal group. However, the expression levels of HMGB1 mRNA in EP treatment group were all significantly decreased at 3 h, 6 h, 12 h and 24 h (2.02 ± 0.27, 2.78 ± 1.16, 3.34 ± 0.31 and 4.21 ± 1.22) as compared with the model group (8.84 ± 2.13) (Figure 3A). Western blot assay further found that the expression of HMGB1 protein was significantly increased in the model group compared with the normal group, but significantly decreased in the EP treatment group at 3 h, 6 h, 12 h and 24 h (2.33 ± 0.67, 2.83 ± 1.07, 3.39 ± 1.01 and 4.02 ± 1.02) compared with the models (7.97 ± 1.85) (Figure 3B). There was no significant difference of HMGB1 expression levels in the EP treatment group at 24 h and 3 h (4.21 ± 1.22 *vs* 2.02 ± 0.27 for HMGB1 mRNA; 4.02 ± 1.02 *vs* 2.33 ± 0.67 for HMGB1 protein) (Figure 3).

Effect of EP on survival

Survival curves of each group are shown in Figure 4. The ACLF rats all died within 3 d without EP treatment, and their median survival time was 60 h. EP treatment at 3 h, 6 h, 12 h and 24 h significantly prolonged the median survival time of the ACLF rats to 162 h, 120 h, 102 h and 78 h, respectively. By log-rank test, there was a significant difference among the curves ($\chi^2 = 41.17$, $P < 0.0001$).

Subcellular localization of HMGB1 expression

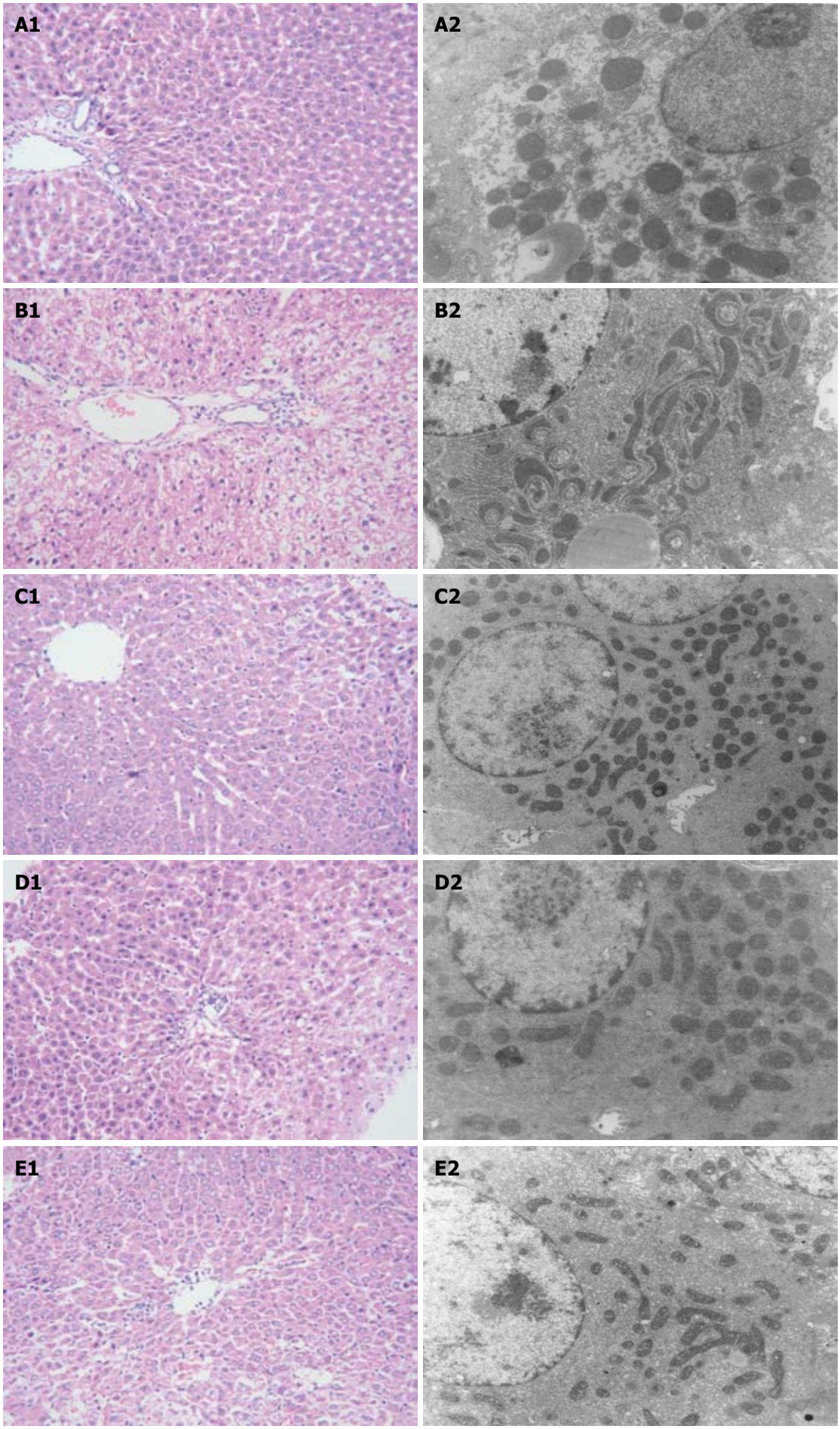
Under confocal laser scanning microscope, HMGB1 expression in liver cells (green fluorescence) was mainly localized in the cytoplasm. A small number of HMGB1 was also observed in the nuclei (Figure 5).

DISCUSSION

The ACLF model has been widely accepted as a good mimic for pathophysiological process and histological characteristics of ACLF[37,38]. In the present study, an ACLF rat model was established firstly by inducing immune liver injury with HSA, and secondly by inducing acute liver failure with D-Gal and LPS treatment. Most of the rats treated with HSA developed cirrhosis and fibrosis, and 90% of them died of acute liver failure after administration of D-Gal and LPS, with a mean survival time of 60 h. Liver histopathology showed massive or submassive necrosis of the regenerated nodules in some areas, where fibrosis septa were intact. Liver function tests were consistent with massive necrosis of hepatocytes. Serum levels of TNF-α and IL-10 increased significantly, a similar result as seen in the patients with ACLF. These results verified that an ACLF model was successfully established, which was further confirmed by pathological characteristics, biochemical data and inflammatory profiles.

HMGB1 protein is involved in the processes of endotoxemia and multiple inflammatory cytokines actively secreted from monocytes/macrophages in the patients and animal models with liver failure[42,43]. Many studies have shown that HMGB1 stimulates the release of inflammatory cytokines in different cell-lines[44-46]. HMGB1 has been also identified as a successful therapeutic target in diverse inflammatory disorders[7,8]. Previously, we demonstrated that the high expression of HMGB1 and the low percentage of Treg cells play an important role in the pathogenesis of liver failure[20]. In the present study, the serum levels of TNF-α, IFN-γ, IL-10, IL-18 and HMGB1 were significantly increased in the ACLF model compared with the normal group. The tissue levels of HMGB1 were also significantly increased and translocations of HMGB1 from nuclei into cytoplasm were presented in ACLF rats. These findings suggested that the increased expression of HMGB1 and/or translocations of HMGB1 were associated with severe liver injury in ACLF, and blocking the release of HMGB1 may represent one of the novel therapeutic strategies for liver failure.

EP has been shown to be beneficial in a variety of animal models of acute and chronic inflammatory conditions[21-28,47,48]. This study investigated whether EP had protective effects on the ACLF model. The histological examinations of liver tissues in ACLF rats showed severe pathological changes at 48 h after induction of


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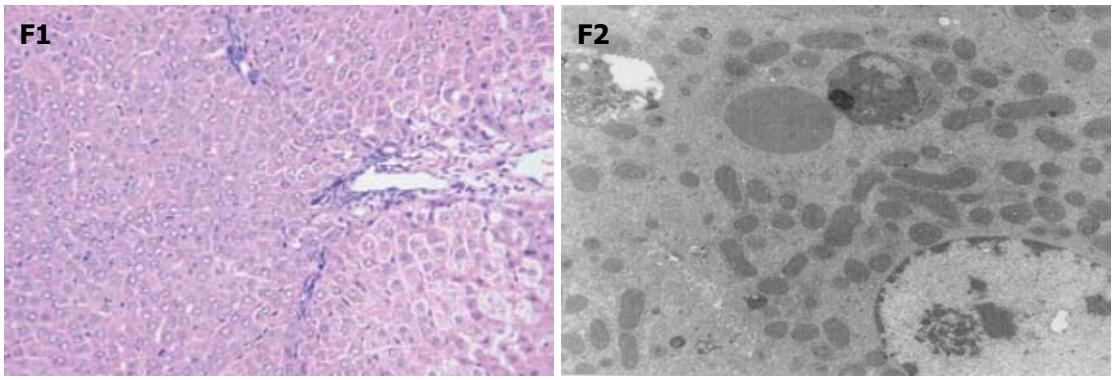


Figure 2 Effect of ethyl pyruvate on liver histology in rat acute-on-chronic liver failure model. A representative image of hematoxylin and eosin (HE) staining (A1, B1, C1, D1) and a representative hepatocyte ultrastructural image (A2, B2, C2, D2) of rat liver tissues. A1, A2: Normal group; B1, B2: Model group; C1, C2: Ethyl pyruvate (EP) treatment at 3 h; D1, D2: EP treatment at 6 h; E1, E2: EP treatment at 12 h; F1, F2: EP treatment at 24 h. HE, original magnification × 200; Electronic microscope, original magnification × 5000.

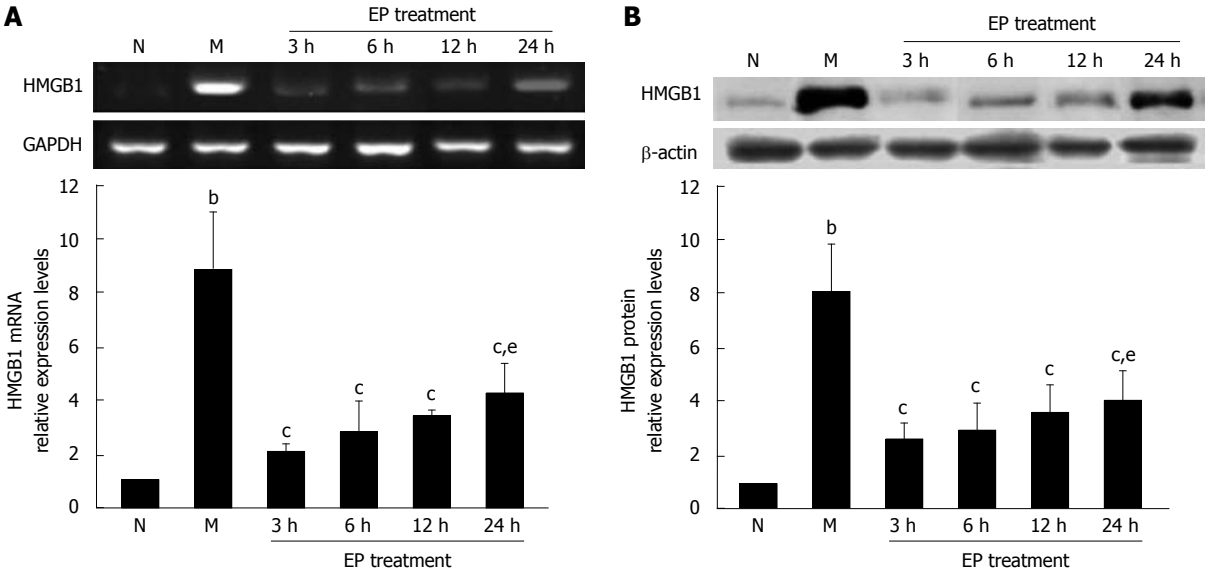


Figure 3 Effect of ethyl pyruvate on high mobility group box-1 expression in liver tissues of rat acute-on-chronic liver failure model. A: A representative reverse transcription-polymerase chain reaction specific for high mobility group box-1 (HMGB1) mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (internal control) in each group shown in the electrophotogram; B: Western blot assay showing the expression of HMGB1 protein in the liver tissues of rats in each group. $^{b}P < 0.01$ *vs* normal; $^{c}P < 0.05$ *vs* model; $^{e}P < 0.05$ *vs* ethyl pyruvate (EP) treatment at 3 h. The time points of 3 h, 6 h, 12 h and 24 h represent four sub-groups of EP treatment group. Results are expressed as mean ± SD ($n = 5$). N: Normal group; M: Model group.

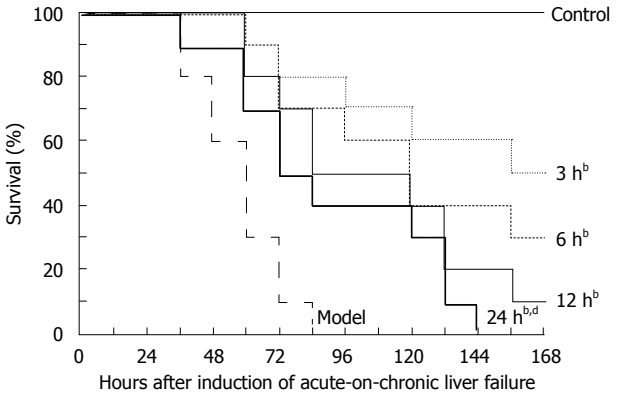


Figure 4 Effect of ethyl pyruvate on survival in rat acute-on-chronic liver failure model. Kaplan-Meier survival curves showed that the median survival time of the model group was 60 h, and the median survival time in ethyl pyruvate treatment group was 162 h, 120 h, 102 h and 78 h, respectively, after treatment at 3 h, 6 h, 12 h and 24 h. By log-rank test, $^{b}P < 0.01$ *vs* model; $^{d}P < 0.01$ *vs* 3 h.

ACLF. However, the hepatic histopathological changes were significantly improved in EP treatment group regardless of treatment time after induction of ACLF. Moreover, serum levels of endotoxin, HMGB1, ALT, TNF-α, IFN-γ, IL-10 and IL-18 were all markedly reduced with EP treatment at different time points. These findings suggested that EP exhibited a protective effect against ACLF rat model through inhibiting the production of inflammatory mediators. Since these inflammatory cytokines, especially the HMGB1, are produced actively by the hepatic macrophage, a major population of monocytes/macrophage lineage, whether EP exerts its inhibitory effects on these inflammatory cytokines through suppressing the hepatic macrophage remains to be further clarified.

A recent study has shown that treatment with anti-HMGB1 antibodies in mice attenuates LPS-induced lethality, even when antibody administration is delayed

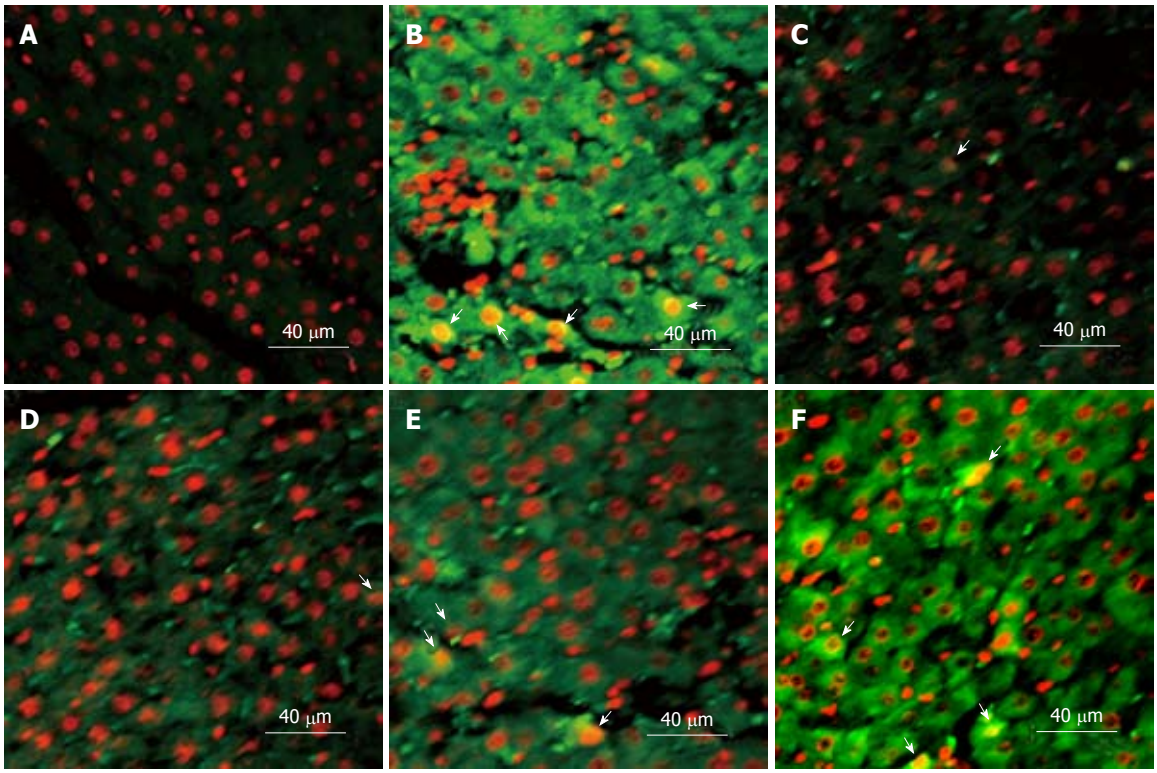


Figure 5 Immunofluorescence and confocal microscopy of high mobility group box-1 expression. Subcellular high mobility group box-1 expression (green fluorescence) was mainly localized in the cytoplasm, and also observed in a small number of nuclei (arrows). A: Normal group; B: Model group; C-F: Ethyl pyruvate treatment at 3 h, 6 h, 12 h and 24 h.

until after the onset of the early proinflammatory cytokine response[6]. In this study, the median survival time of the ACLF rats was 60 h. But EP treatment at 3 h, 6 h, 12 h and 24 h significantly prolonged the median survival time of the model rats to 162 h, 120 h, 102 h and 78 h, respectively, after induction of ACLF. The results showed that HMGB1 expressions in both serum and liver tissues were effectively inhibited in EP treatment group, accompanied with a significant decrease of endotoxin and improvement of hepatic histopathology. In EP treatment group, translocations of HMGB1 from nuclei into cytoplasm were markedly reduced depending on the treatment time points. The inhibitory effect of EP on HMGB1 at 3 h was better than at 24 h after induction of ACLF. It may indicate that early EP treatment could result in more obvious improvement in ACLF rats. However, EP treatment at 24 h after induction of ACLF also improved the liver histological changes and prolonged the survival of ACLF rats. It suggests that EP, as a HMGB1 inhibitor, could improve the ACLF outcome, even when HMGB1 inhibitor administration was delayed until induction of ACLF and after the onset of the early proinflammatory factor response. This indicates that targeted therapy against HMGB1 could be initiated even in the later stage of acute inflammation. It would provide a wider therapeutic time window for the treatment of endotoxemia in ACLF.

In summary, the present study demonstrates that EP administration can inhibit HMGB1 expression, decrease endotoxin level, reduce secretion of inflammatory cytokines, ameliorate hepatocellular injury, improve survival, and protect against ACLF in rats. These findings suggest that EP may be a potential and novel therapeutic agent for severe liver inflammatory injury.

COMMENTS

Background

Acute-on-chronic liver failure (ACLF) is a severe life-threatening condition, for which no effective treatment is available. More and more evidences have indicated that intestinal endotoxemia plays a significant role in the pathogenesis and progression of liver failure. A growing number of studies have also shown that high mobility group box-1 (HMGB1), as an important inflammatory mediator of endotoxemia, is a successful therapeutic target in experimental models of severe inflammatory diseases.

Research frontiers

Ethyl pyruvate (EP), an inhibitor of HMGB1, has been shown to improve survival and ameliorate organ dysfunction in a variety of animal models of critical illness. However, whether EP could also be a good candidate for the treatment of liver failure remains unknown. In this study, an ACLF rat model was treated with EP at different time points. The effects of EP on liver failure were investigated by observing the changes of various parameters, including endotoxin, HMGB1, liver function, cytokines, liver histology and animal survival.

Innovations and breakthroughs

The most common clinical cause of liver failure in Asia, especially in China, is an acute episode of liver function decompensation on the basis of an already impaired liver function caused mostly by chronic hepatitis B virus infection, which is defined as ACLF. Therefore, the traditional animal models of ACLF induced directly by D-galactosamine (D-Gal) or lipopolysaccharide (LPS) in previous studies can not simulate the pathophysiological process of ACLF. In this study, the authors employed an ACLF rat model which was established firstly

by human serum albumin treatment to induce immune liver injury, and secondly by administration of D-Gal and LPS to induce acute liver failure on the basis of chronic liver injury. Since this animal model can well simulate part of the pathophysiological processes of acute liver failure on the basis of chronic liver diseases, the experimental results of treatment with EP turned out to be more persuasive. This study showed that EP administration can inhibit HMGB1 expression, decrease endotoxin level, reduce secretion of inflammatory cytokines, ameliorate hepatocellular injury, and improve the survival of the ACLF rats.

Applications

The study results suggest that EP may be a potential and novel therapeutic agent for severe liver inflammatory injury.

Terminology

ACLF is defined as acute hepatic insult manifesting as jaundice and coagulopathy, complicated within 4 wk with ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease according to the consensus recommendations of the Asian Pacific Association for the Study of the Liver; HMGB1 protein, as a late inflammatory mediator, can be released into the extracellular milieu from activated macrophages, immuno-stimulated gut epithelial cells and necrotic or damaged cells in a delayed manner relative to the secretion of other proinflammatory mediators, such as tumor necrosis factor-α and interleukin-1, and contributes to the pathogenesis of diverse inflammatory and infectious disorders; EP, which is a simple aliphatic ester derived from pyruvic acid and is synthesized from pyruvate and ethanol, is stable in Ringer's EP solution through interactions with calcium and potassium ions. Treatment with EP has been shown to be beneficial in animal models of a variety of acute and chronic inflammatory conditions.

Peer review

This is a very well designed study and the literature relating to the rationale for using this compound in this animal model to determine its potential effects has been thoroughly explored. The study is well presented in terms of its goals and purposes. There is convincing data to suggest that EP may be explored as a potential therapeutic agent for severe liver diseases.

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ORIGINAL ARTICLE

Exogenous carbon monoxide attenuates inflammatory responses in the small intestine of septic mice

Xu Wang, Jie Cao, Bing-Wei Sun, Da-Dong Liu, Feng Liang, Liang Gao

Xu Wang, Jie Cao, Bing-Wei Sun, Da-Dong Liu, Feng Liang, Liang Gao, Department of Burns and Plastic Surgery, Affiliated Hospital, Jiangsu University, Zhenjiang 212001, Jiangsu Province, China

Author contributions: Wang X, Cao J and Sun BW designed research; Cao J and Liu DD contributed new reagents/analytic tools; Sun BW, Liang F and Cao L analyzed data; Sun BW wrote the paper.

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Correspondence to: Dr. Bing-Wei Sun, Department of Burns and Plastic Surgery, Affiliated Hospital, Jiangsu University, 438 Jiefang Road, Zhenjiang 212001, Jiangsu Province, China. sunbinwe@hotmail.com
Telephone: +86-511-85082258 Fax: +86-511-5029089

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Abstract

AIM: To determine whether the carbon monoxide (CO)-releasing molecules (CORM)-liberated CO suppress inflammatory responses in the small intestine of septic mice.

METHODS: The C57BL/6 mice (male, n = 36; weight 20 ± 2 g) were assigned to four groups in three respective experiments. Sepsis in mice was induced by cecal ligation and puncture (CLP) (24 h). Tricarbonyldichlororuthenium (Ⅱ) dimer (CORM-2) (8 mg/kg, *i.v.*) was administrated immediately after induction of CLP. The levels of inflammatory cytokines [interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α)] in tissue homogenates were measured with enzyme-linked immunosorbent assay. The levels of malondialdehyde (MDA) in the tissues were determined. The levels of nitric oxide (NO) in tissue homogenate were measured and the expression levels of intercellular adhesion molecule 1 (ICAM-1) and inducible nitric oxide synthase (iNOS) in the small intestine were also assessed. NO and IL-8 levels in the supernatants were determined after the human adenocarcinoma cell line Caco-2 was stimulated by lipopolysaccharide (LPS) (10 g/mL) for 4 h *in vitro*.

RESULTS: At 24 h after CLP, histological analysis showed that the ileum and jejunum from CLP mice induced severe edema and sloughing of the villous tips, as well as infiltration of inflammatory cells into the mucosa. Semi-quantitative analysis of histological samples of ileum and jejunum showed that granulocyte infiltration in the septic mice was significantly increased compared to that in the sham group. Administration of CORM-2 significantly decreased granulocyte infiltration. At 24 h after CLP, the tissue MDA levels in the mid-ileum and mid-jejunum significantly increased compared to the sham animals (103.68 ± 23.88 nmol/mL *vs* 39.66 ± 8.23 nmol/mL, 89.66 ± 9.98 nmol/mL *vs* 32.32 ± 7.43 nmol/mL, $P < 0.01$). *In vitro* administration of CORM-2, tissue MDA levels were significantly decreased (50.65 ± 11.46 nmol/mL, 59.32 ± 6.62 nmol/mL, $P < 0.05$). Meanwhile, the tissue IL-1β and TNF-α levels in the mid-ileum significantly increased compared to the sham animals (6.66 ± 1.09 pg/mL *vs* 1.67 ± 0.45 pg/mL, 19.34 ± 3.99 pg/mL *vs* 3.98 ± 0.87 pg/mL, $P < 0.01$). *In vitro* administration of CORM-2, tissue IL-1β and TNF-α levels were significantly decreased (3.87 ± 1.08 pg/mL, 10.45 ± 2.48 pg/mL, $P < 0.05$). The levels of NO in mid-ileum and mid-jejunum tissue homogenate were also decreased (14.69 ± 2.45 nmol/mL *vs* 24.36 ± 2.97 nmol/mL, 18.47 ± 2.47 nmol/mL *vs* 27.33 ± 3.87 nmol/mL, $P < 0.05$). The expression of iNOS and ICAM-1 in the mid-ileum of septic mice at 24 h after CLP induction significantly increased compared to the sham animals. *In vitro* administration of CORM-2, expression of iNOS and ICAM-1 were significantly decreased. In parallel, the levels of NO and IL-8 in the supernatants of Caco-2 stimulated by LPS was markedly decreased in CORM-2-treated Caco-2 cells (2.22 ± 0.12 nmol/mL *vs* 6.25 ± 1.69 nmol/mL, 24.97 ± 3.01 pg/mL *vs* 49.45 ± 5.11 pg/mL, $P < 0.05$).

CONCLUSION: CORM-released CO attenuates the inflammatory cytokine production (IL-1β and TNF-α), and suppress the oxidative stress in the small intestine during sepsis by interfering with protein expression of ICAM-1 and iNOS.

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Key words: Sepsis; Small intestine; Inflammation; Carbon monoxide

Peer reviewer: Alireza Mani, MD, PhD, Department of Physiology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran 14115-331, Iran

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INTRODUCTION

Sepsis is a complex clinical syndrome resulting from a harmful host inflammatory response to infection. Despite advancements in understanding the pathophysiology of sepsis, clinical outcomes are variable and the mortality rate remains high among the patients[1]. Cecal ligation and puncture (CLP) may induce the activation of an inflammatory cascade, leading to sepsis and multiple organ failure[2]. Some reports have indicated that the inflammatory response syndrome, which contributes to oxidative cell/tissue damage, might frequently be accompanied by leukocyte sequestration in many important organ systems in the body[3]. The increase of production of pro-inflammatory mediators such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) is closely associated with activation of leukocytes and macrophages which were sequestrated in the tissue[4,5]. Leukocyte sequestration and its subsequent infiltration in organ tissue can cause leukocyte activation and contribute to vascular damage and the development of systemic inflammatory reaction. As the prerequisite, activation of leukocytes and endothelial cells results in aggregation of leukocytes, platelets and erythrocytes *in vitro*. This may favor disseminated intravasal coagulation and further multiple organ failure.

It has been shown that endogenous carbon monoxide (CO), a bi-product of inducible heme oxygenase (HO-1) can modulates inflammation. Recent studies suggest that exogenously administered CO inhibits lipopolysaccharide (LPS)-induced production of cytokines both *in vivo* and *in vitro*, and consequently exhibits an important cytoprotective function and anti-inflammatory properties which benefit for the resolution of acute inflammation[6].

Recently, transitional metal carbonyls have been identified as potential CO-releasing molecules (CORMs) with the potential to facilitate the pharmaceutical use of CO by delivering it to tissues and organs[7]. Our previous studies[8-14] firstly confirmed that CORM-released CO attenuated leukocytes sequestration in the liver, lung and small intestine of burned mice by interfering with nuclear factor-κB (NF-κB) activation, protein expression of intercellular adhesion molecule-1 (ICAM-1) and therefore suppressing endothelial cells pro-adhesive phenotype. However, to date, little is known if CORM-released CO can down-regulate the inflammatory production and oxidative stress in the small intestine of the septic mice. This study aims to investigate the protective effects and the underlying mechanisms of tricarbonyldichlororuthenium (Ⅱ) dimer (CORM-2), one of the novel groups of CORMs, on the suppression of inflammatory cytokine production and malondialdehyde (MDA) levels as an indicator of oxidative stress index in the small intestine of septic mice induced by CLP.

MATERIALS AND METHODS

Materials

CORM-2 was obtained from Sigma Aldrich and solubilized in dimethyl sulfoxide (DMSO) to obtain a 10 mmol/L stock. Inactive form of the compound (negative control) was also used in some experiments and it was prepared as follows: CORM-2 was "inactivated" (iCORM-2) by adding the compound to DMSO and kept for 18 h at 37 ℃ in a 5% CO_2 humidified atmosphere to liberate CO. The iCORM-2 solution was finally bubbled with nitrogen to remove the residual CO present in the solution. Cell culture reagents were obtained from Gibco (Grand Island, NY) and culture supplies from Corning (Corning, NY) and Falcon (Lincoln Park, NJ). Polyclonal antibody against ICAM-1 and inducible nitric oxide synthase (iNOS) was purchased from Santa Cruz Biotechnology Inc., United States. All the other chemicals were of reagent grade and obtained from Sigma unless otherwise stated.

Animal and CLP protocol

The C57BL/6 mice (male, n = 36; body weight 20 ± 2 g) were fed a standard laboratory diet and water ad libitum. Mice were assigned to four groups in three respective experiments. In each experiment, mice in sham group (n = 9) were underwent sham procedure, whereas mice in CLP group (n = 9) received cecal ligation and puncture, mice in CORM-2 group (n = 9) and iCORM group (n = 9) subjected to the same injury with immediate administration of CORM-2 (8 mg/kg, *i.v.*) and iCORM-2 (8 mg/kg, *i.v.*), respectively. The concentration of CORM-2 used in the present study was based on a previous report in of the use of this compound in mice[15] and the preliminary experiments in our lab[8,9]. The experimental protocol was approved by the Council on Animal Care at Jiangsu University on the Protection and the Welfare of Animals and followed the National

Institutes of Health guidelines for the care and use of experimental animals.

Mice were anesthetized with 2% isoflurane in oxygen *via* a facemask. A 2-cm midline incision was made through the abdominal wall; the cecum was identified and ligated with a 3-0 silk tie 1 cm from the tip. Care was taken not to cause bowel obstruction. A single puncture of the cecal wall was performed with a 20-gauge needle. The cecum was lightly squeezed to express a small amount of stool from the puncture site to assure a full-thickness perforation. Great care was taken to preserve the continuity of flow between the small and large bowels. The cecum was returned to the abdominal cavity, and the incision was closed with surgiclips. Sham mice underwent midline laparotomy under anesthesia; the cecum was exteriorized and returned to the abdomen, and the incision was closed with surgiclips. The animals were sacrificed at 24 h after experimental manipulation.

Histologic studies

The mid-ileum and mid-jejunum specimens harvested from different groups of animals were immersed in 4% formaldehyde solution at 24 h after CLP. The tissue was embedded in paraffin wax, serially sectioned, and stained with hematoxylin-eosin. Tissue morphologic characteristics were evaluated under light microscope. Ileum and jejunum tissues were evaluated for density of granulocytes and degree of hydropic degeneration. Tissues were evaluated in a semi-quantitatively manner by two experienced independent examiners who were blinded to the experimental groups. A scoring system was used for each item using 0 up to 2 points for the different states of organ damage (with 2 being most granulocytes, edema and degeneration). Afterwards, the mean ± SE of each item was calculated.

Preparation of tissue homogenates

The intestine was exposed at 24 h after CLP. Retaining approximately the first 5 cm-long proximal segment of intestine, 3 cm-long segments of jejunum and ileum were removed, cleaned, and snap-frozen in liquid nitrogen. The samples were stored at -70 ℃. Equal weights (100 mg wet weight) of intestine from various groups were suspended in 1 mL phosphate buffered saline and sonicated on ice (30 cycles, twice for 30 s)[8,9]. Homogenates were cleared by centrifuging at 12 000 *r*/min at 4 ℃, and the supernatants were stored at -70 ℃. Protein levels in the homogenates were determined using the Bio-Rad (Hercules, CA) assay kit.

Measurement of IL-1β and TNF-α

The levels of the inflammatory cytokines IL-1β and TNF-α in ileum and jejunum tissue homogenates were measured using enzyme-linked immunosorbent assay kits (R and D Systems, Minneapolis, MN) following the manufacturers instructions.

Cell culture and stimulation

Caco-2, the human adenocarcinoma cell line, was obtained from American Tissue Culture Collection (ATCC, Rockville, MD, United States). The cells were passaged and grown in Dulbecco's modified Egle's medium (DMEM) supplemented with 1% nonessential amino acids, 10% fetal calf serum, 1 mg/L streptomycin, and 1000 U/L penicillin (Complete DMEM). Experiments were performed with cell monolayers that were in culture for 21-25 d. Cells were seeded in the collagen-coated 48-well plates with the same cell density. Medium was replaced every day when each experiment began. Cells were cultured at 37 ℃, 5% CO_2, and 90% related humidity. The supernatants of Caco-2 cell stimulated by LPS (10 g/mL) for 4 h were collected for nitric oxide (NO) and IL-8 levels measurement.

Measurement of NO in tissue homogenate and supernatants of Caco-2 cells

NO was measured as described previously[11]. Briefly, 100 L of supernatants was removed and incubated with 100 L of Griess reagent (10 g/L sulfanilamide and 1 g/L N-1-naphthylethylenediamine dihydrochloride in 25 mL/L phosphoric acid) in a 96-well plate. The plate was incubated for 10 min at room temperature. Nitrite production was quantified spectrophotometrically using an automated colorimetric procedure. Absorbance at 540 nm was measured using a microplate reader (Bio-Tek, United States). The nitrite concentration was calculated by comparing samples with standard solutions of sodium nitrite produced in the culture medium. All samples were assayed in triplicate.

MDA assay

MDA formation was utilized as a lipoperoxidation index. The levels of MDA in the small intestines were determined as described by Sun *et al*[10]. Briefly, tissue was homogenized in 1.15% KCl solution (1:10 volume). An aliquot (0.1 mL) of the homogenate was added to a reaction mixture [200 L of 8.1% SDS, 1500 L of 20% acetic acid (pH 3.5), 1500 L of 0.8% thiobarbituric acid, and 700 L distilled water]. Samples were then boiled for 1 h at 95 ℃ and centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 532 nm (Jenway, Mod. 6300, Dunmow, Essex, United Kingdom). Data were expressed in nmol per milligram of wet tissue.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured according to the established method[8,9]. Briefly, tissue was homogenized in 0.5 mL of 50 mmol/L potassium phosphate buffer (pH 7.4) and centrifuged at 10 000 × *g* at 4 ℃ for 30 min. The remaining pellet was resuspended in 0.5 mL of 50 mmol/L potassium buffer at pH 6.0 with 0.5% hexadecyltrimethylammonium bromide, sonicated on ice, and then centrifuged at 12 000 × *g* at 4 ℃ for 10 min. Supernatants were then assayed at a 1:20 dilution in reaction buffer containing 50 mmol/L phosphate buffer, 530 mmol/L o-dianisidine, and 20 mmol/L H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO that caused a change in absor-

Table 1 Histological scoring of granulocyte infiltration and hydropic degeneration in ileum and jejunum tissue stained with hematoxylin-eosin 24 h after cecal ligation and perforation

	Granulocyte infiltration		Hydropic degeneration	
	Ileum	Jejunum	Ileum	Jejunum
Sham	0.50 ± 0.12	0.51 ± 0.11	0	0
CLP	1.90 ± 0.46[a]	1.67 ± 0.12[a]	1.79 ± 0.34[b]	1.68 ± 0.28[b]
CLP + CORM	0.98 ± 0.12[c]	0.79 ± 0.34[c]	1.67 ± 0.44	1.72 ± 0.36
CLP + iCORM	1.88 ± 0.30[a]	1.61 ± 0.17[a]	1.76 ± 0.67[b]	1.62 ± 0.45[b]

For granulocyte infiltration: 0, no granulocyte infiltration; 1, moderate granulocyte infiltration; 2, severe granulocyte infiltration. For hydropic degeneration: 0, no hydropic degeneration; 1, moderate hydropic degeneration; 2, severe hydropic degeneration. Values are presented as mean ± SE. [a]$P < 0.05$, [b]$P < 0.01$ *vs* sham group; [c]$P < 0.05$ *vs* cecal ligation and perforation (CLP) group. CORM: Carbon monoxide-releasing molecule; iCORM: Inactivated-carbon monoxide-releasing molecule.

bance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Western blot analysis

Tissues were homogenized for extract preparations in ice-cold mild lysis buffer, containing 1% Nonidet P-40, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate (pH 7.2), 2 mmol/L ethylene diamine tetraacetic acid, 50 mmol/L sodium fluoride, 0.2 mmol/L sodium vanadate, and 1 g/mL of aprotinin. The tissue homogenates were centrifuged at 20 000 × *g* for 15 min and supernatants were collected. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed on an equivalent amount of protein samples using precast 7% resolving/4% stacking Tris-HCl gels (Bio-Rad, Hercules, CA). Separated proteins were then transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Membranes were blocked in 5% nonfat milk in TBS buffer containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Blocked membranes were incubated in primary antibody specific for mouse iNOS and ICAM-1 at a concentration of 1:2000 and 1:5000, respectively in TBST overnight at 4 ℃. Then the membranes were washed and probed with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) for 1 h at room temperature. Chemiluminescence detection was performed with the Amersham enhanced chemiluminescence detection kit according to the manufacturer's instructions. To ensure a similar amount of protein in each sample, the membranes were "stripped off", reprobed with actin, developed with horseradish peroxidase-conjugated secondary antibody, and visualized by enhanced chemiluminescence.

Statistical analysis

All the values were presented as mean ± SE. Statistical analysis was performed using the analysis of variance and Student's *t*-test for the comparisons. $P < 0.05$ was considered to be statistically significant.

RESULTS

Histology

Histological analysis showed that the ileum and jejunum from sham mice had the normal architecture of the intestinal epithelium and wall, while CLP induced severe edema and sloughing of the villous tips, as well as infiltration of inflammatory cells into the mucosa. Semiquantitative analysis of histological samples of ileum and jejunum showed that the granulocyte infiltration in the septic mice was significantly increased compared with that in the sham group. Administration of CORM-2 (8 mg/kg, *i.v.*), significantly decreased granulocyte infiltration. However, CORM-2 did not improve the hydropic degeneration induced by sepsis in either the ileum or jejunum (Figure 1 and Table 1).

Effect of CORM-2 on MPO activity in small intestine of septic mice

To determine whether the CLP-induced increase in polymorphonuclear neutrophil (PMN) accumulation in the small intestine was effectively prevented by CORM-2, the activity of MPO, an enzyme in azurophilic granules of neutrophils, was assessed. Extracts of the ileum and jejunum samples were examined for content of MPO 24 h after sepsis. The mean MPO levels are shown in Figure 2. MPO activity in organs obtained from septic mice was markedly increased compared with that in the sham group ($P < 0.01$), while it was significantly decreased by treatment with CORM-2 ($P < 0.05$).

Effect of CORM-2 on tissue MDA in small intestine of septic mice

Tissue MDA levels are considered important markers of lipoperoxidation associated to oxidative stress. The mean MDA levels detected in the mid-ileum and mid-jejunum of mice were significantly affected by CLP injury. At 24 h after CLP, the tissue MDA levels in the mid-ileum and mid-jejunum significantly increased compared with the sham animals. After *in vitro* administration of CORM-2 (8 mg/kg, *i.v.*), tissue MDA levels were significantly decreased (Figure 3A).

Effect of CORM-2 on TNF-α and IL-1β levels in tissue homogenates of septic mice

As shown in Figure 3A, at 24 h after CLP, the expression of TNF-α and IL-1 in mid-ileum and mid-jejunum homogenates of CLP-challenged mice was markedly increased compared with the sham mice. After administration of CORM-2, the elevation levels of TNF-α and IL-1β in tissue homogenates were significantly diminished (Figure 3B and C).

Effect of CORM-2 on NO production in tissue homogenates of septic mice

As shown in Figure 3D, production of nitrite was low in sham group. After CLP challenge, nitrite levels in tissue

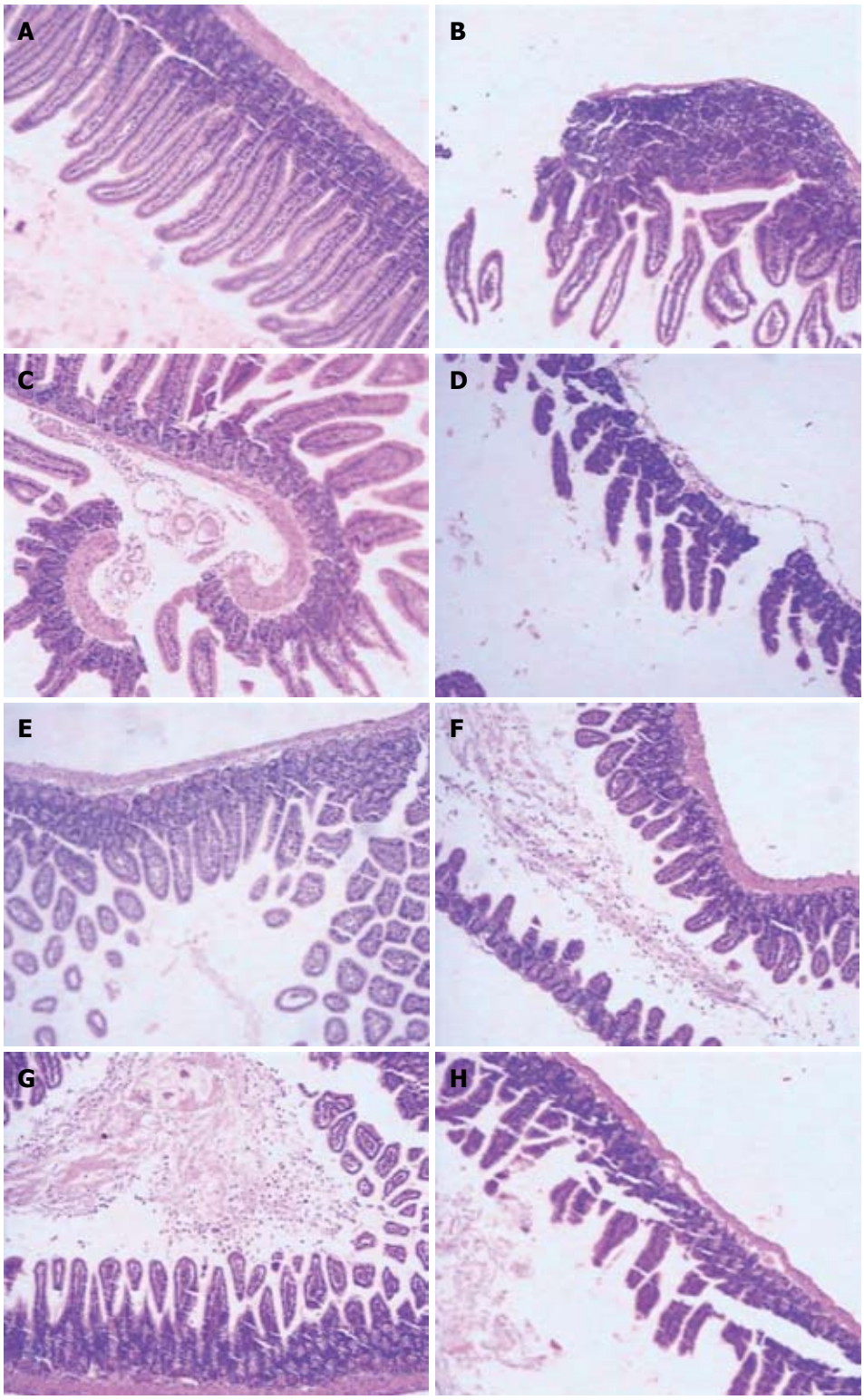


Figure 1 Effects of tricarbonyldichlororuthenium (Ⅱ) dimer carbon monoxide-releasing molecules on small intestine injury in septic mice. Mice were challenged with cecal ligation and perforation (CLP) and treated with tricarbonyldichlororuthenium (Ⅱ) dimer. The mid-ileum (A-D) and mid-jejunum (E-H) specimens harvested from different groups of animals were immersed in 4% formaldehyde solution at 24 h after CLP. The tissue was embedded in paraffin wax, serially sectioned, and stained with hematoxylin-eosin. Tissue morphologic characteristics were evaluated by light microscopy. A, E: Sections from sham mice had normal architecture of the intestinal epithelium and wall; B, F: Sections from septic mice showed inflammatory cell infiltration through the wall, concentrated below the epithelial layer, edema of the distal portion of the villi, and necrosis of the epithelium at the villous tips; C, G: Section from septic mice treated with carbon monoxide-releasing molecules-2 showed a significant decrease in granulocyte infiltration, while no marked improvement of hydropic degeneration. The figure is representative of at least three experiments performed on different days.

homogenates were significantly increased ($P < 0.05$ *vs* sham group). However, nitrite levels were markedly decreased in the CORM-2 group compared with the CLP group ($P < 0.05$).

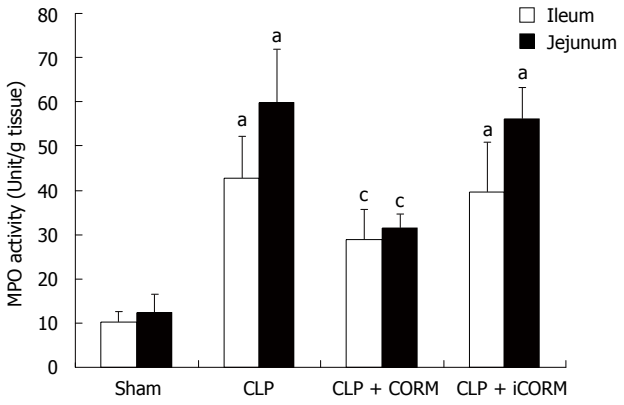


Figure 2 Effects of tricarbonyldichlororuthenium (Ⅱ) dimer carbon monoxide-releasing molecules on myeloperoxidase activity in the small intestine of septic mice. Mice were challenged with cecal ligation and perforation (CLP) and treated with tricarbonyldichlororuthenium (Ⅱ) dimer. Myeloperoxidase (MPO) activity in the mid-ileum and mid-jejunum were assessed 24 h following CLP injury. Results are mean ± SE, $^{a}P < 0.01$ *vs* sham mice; $^{c}P < 0.05$ *vs* CLP mice. CORM: Carbon monoxide-releasing molecule; iCORM: Inactivated-carbon monoxide-releasing molecule.

Effect of CORM-2 on IL-8 and NO production in supernatants of LPS- stimulated Caco-2 cells

Production of IL-8 and nitrite was low in the control Caco-2 cells. After stimulation of LPS, IL-8 and nitrite levels were significantly increased ($P < 0.05$ *vs* control group). CORM-2 significantly reduced IL-8 and nitrite generation in the LPS-stimulated Caco-2 cells ($P < 0.05$ *vs* CLP group, Figure 4).

Effect of CORM-2 on expression of ICAM-1 and *iNOS in the small intestine of septic mice*

The expression of iNOS and ICAM-1 in the mid-ileum of septic mice at 24 h after CLP induction was significantly enhanced compared with the sham animals. After *in vitro* administration of CORM-2 (8 mg/kg, *i.v.*), the expression of iNOS and ICAM-1 was significantly decreased (Figure 5).

DISCUSSION

Sepsis, which frequently occurs after hemorrhage, trauma, burn, or abdominal surgery, continues to be a clinical challenge in clinic as a leading cause of morbidity and mortality in severely ill patients[1,16-19]. Despite the therapeutic strategies focusing on local host defenses and the inhibition of overwhelming inflammation response, little progress has been achieved. CLP may induce the activation of an inflammatory cascade, and cause damage to multiple organs, leading to sepsis and multiple organ failure. This model seems to resemble qualitatively as well as quantitatively the clinical observations of vascular reactivity and inflammation in the course of polymicrobial peritonitis, bacteremia, and systemic sepsis[20-23]. Therefore, this study aims to evaluate the possible role of CORM-derived CO in CLP-induced sepsis.

During sepsis, the most frequent complications within the gastrointestinal tract are small intestinal and mucosal barrier dysfunctions. Our previous data indicated that the intestine is one of the tissues most sensitive to the ischemia and reperfusion (I/R) induced by thermal injury[8-10], and a novel metal carbonyl-based compound (CORM-2) which exerts a protective effect against the pathological changes caused by thermal injury of the small intestine. In this study, we employed the same compound to determine whether it suppresses inflammatory cytokine production and oxidative stress in the small intestine of the septic mice.

Leukocyte sequestration and its subsequent infiltration in tissues can cause leukocyte activation and contribute to vascular damage and the development of systemic inflammatory reaction. MPO is an enzyme that is found predominantly in the azurophilic granules of PMN. Tissue MPO activity is frequently utilized to estimate tissue PMN accumulation in damaged tissues and correlates significantly with the number of PMN determined histochemically in tissues[24-26]. In the present study, we found that MPO activities in small intestine were markedly enhanced after CLP and *in vitro* administration of CORM-2 resulted in significantly downregulation of MPO activity. The direct cause of leukocytes sequestration after CLP is considered to be the higher expression of adhesion molecules (ICAM-1). ICAM-1 activates leukocytes and endothelial cells, which in turn prompt the release of various inflammatory mediators, resulting in systemic inflammatory response syndrome, acute respiratory distress syndrome and multiorgan dysfunction syndrome[27]. At 24 h after CLP, the expression of ICAM-1 in small intestine was markedly upregulated. *In vitro* administration of CORM-2 inhibited the upregulation of ICAM-1 induced by CLP. These findings indicated that CORM-2 can effectively prevent PMN chemotaxis and infiltration in the tissues after CLP, consequently decreasing the production of oxidants and reducing tissue oxidative injury.

Pro-inflammatory cytokines, such as TNF-α and IL-1 have been shown to be released early after an inflammatory stimulus. TNF-α is a pleiotropic cytokine with strong proinflammatory and immunomodulatory properties, and plays a critical role in inflammation and inflammatory bowel disease[28,29]. Various strategies have been explored to inhibit TNF-α. To confirm if prevention of sepsis-induced intestinal dysmotility by CORM-2 was partly through interruption of the cycle of inflammatory events in the local intestine, we investigated the expression of inflammatory cytokines TNF-α in the small intestine of the septic mice. We observed marked increases in TNF-α levels in the tissue homogenates of ileum and jejunum after CLP injury. *In vitro* administration of CORM-2 was able to inhibit the inflammatory production in enteric tissue induced by CLP. Our findings strongly indicated that CORM-2 appears to inhibit upregulation of inflammatory production, and consequently might effectively decrease inflammatory response in the small intestine induced by CLP. Similarly,

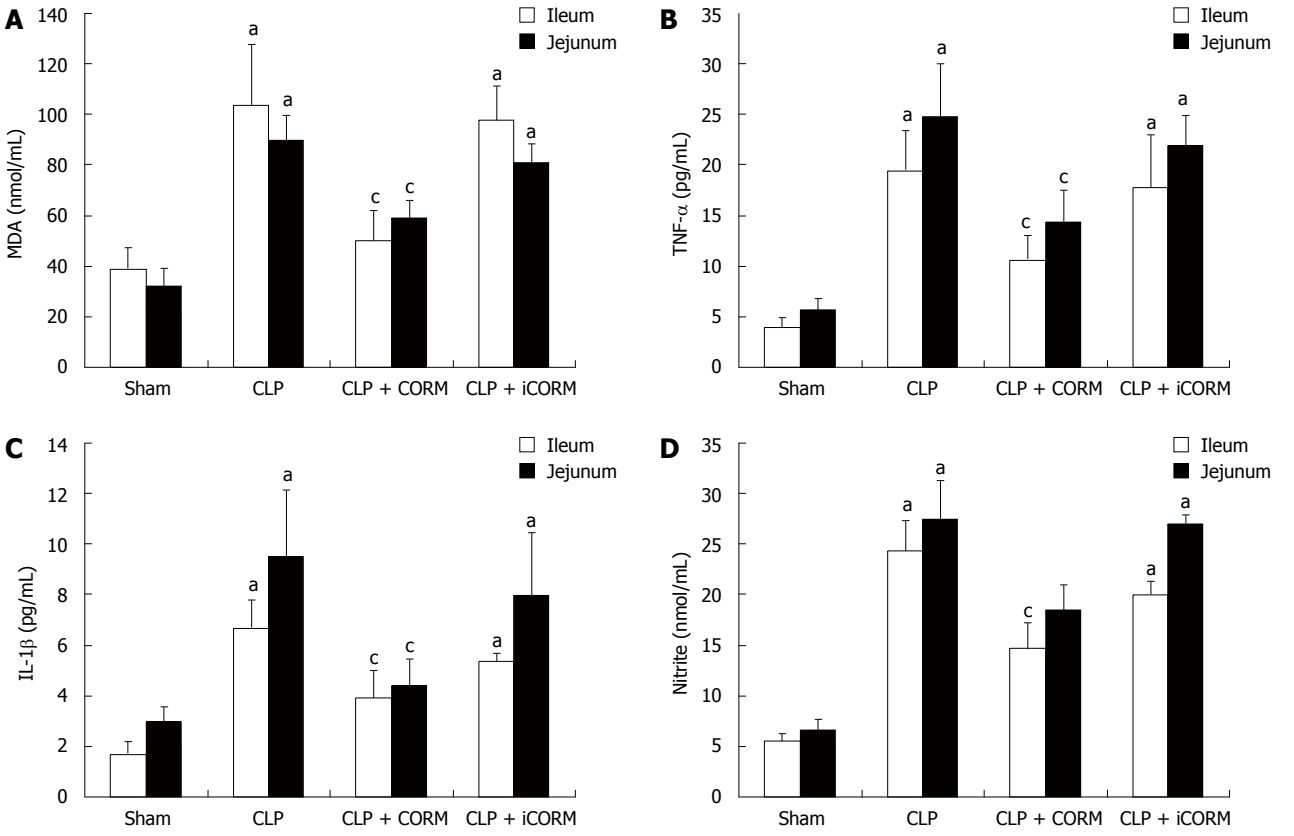


Figure 3 Effects of tricarbonyldichlororuthenium (Ⅱ) dimer carbon monoxide-releasing molecules on malondialdehyde, expression of interleukin-1β and tumor necrosis factor-α and nitrite production in the mid-ileum and mid-jejunum of septic mice. A: Mice were challenged with cecal ligation and perforation (CLP) and treated with tricarbonyldichlororuthenium (Ⅱ) dimer. Malondialdehyde (MDA) in the mid-ileum and mid-jejunum was assessed 24 h following CLP injury; B: Tumor necrosis factor (TNF)-α levels in the mid-ileum and mid-jejunum was assessed 24 h following CLP injury; C: Interleukin (IL)-1β levels in the mid-ileum and mid-jejunum was assessed 24 h following CLP injury; D: Nitrite production in the mid-ileum and mid-jejunum was assessed 24 h following CLP injury. Results are mean ± SE, $^{a}P < 0.01$ *vs* sham mice; $^{c}P < 0.05$ *vs* CLP mice. CORM: Carbon monoxide-releasing molecule; iCORM: Inactivated-carbon monoxide-releasing molecule.

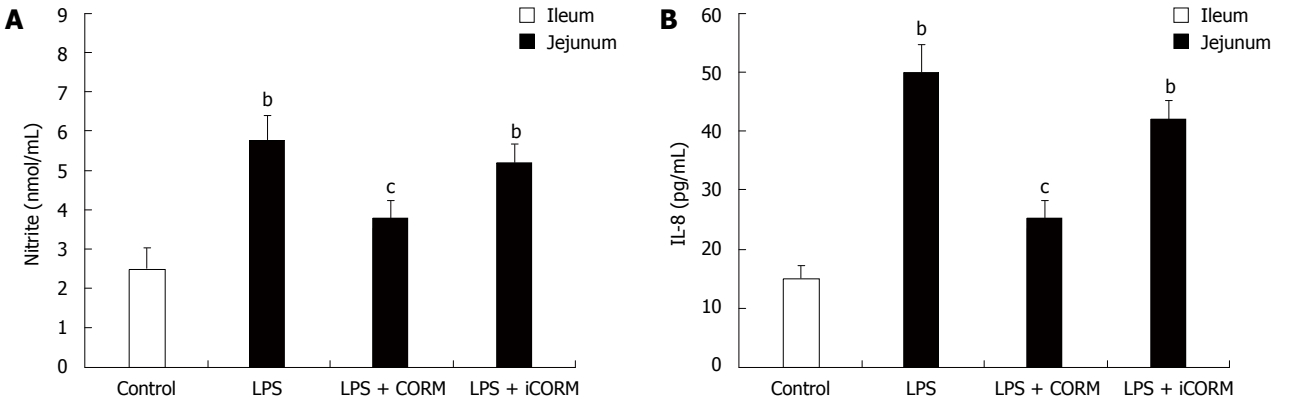


Figure 4 Effects of tricarbonyldichlororuthenium (Ⅱ) dimer carbon monoxide-releasing molecules on interleukin-8 and nitrite production in Caco-2 cells. Caco-2 cells were stimulated by lipopolysaccharide (LPS) and treated with tricarbonyldichlororuthenium (Ⅱ) dimer. Nitrite (A) and interleukin (IL)-8 (B) production in supernatants of Caco-2 cells were assessed 4 h following LPS stimulation. Results are mean ± SE. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* LPS-stimulated Caco-2. CORM: Carbon monoxide-releasing molecule; iCORM: Inactivated-carbon monoxide-releasing molecule.

another important cytokine, IL-1, was also found to be markedly upregulated in ileum and jejunum of septic mice. Downregulation of IL-1 levels was most remarkable in the small intestine of septic mice treated with CORM-2. These findings demonstrate that treatment with CORM-2 suppresses the production of IL-1 and TNF-α, and subsequently attenuates the inflammatory response induced by CLP in the small intestine.

Tissue MDA content, the last product of lipid breakdown caused by oxidative stress, is considered to be a good indicator of radical-induced lipid peroxidation[29]. Increased lipid peroxidation in the small intestine of animals with intestinal I/R, was evidenced by significantly increased MDA levels[30]. In the present study, intestinal

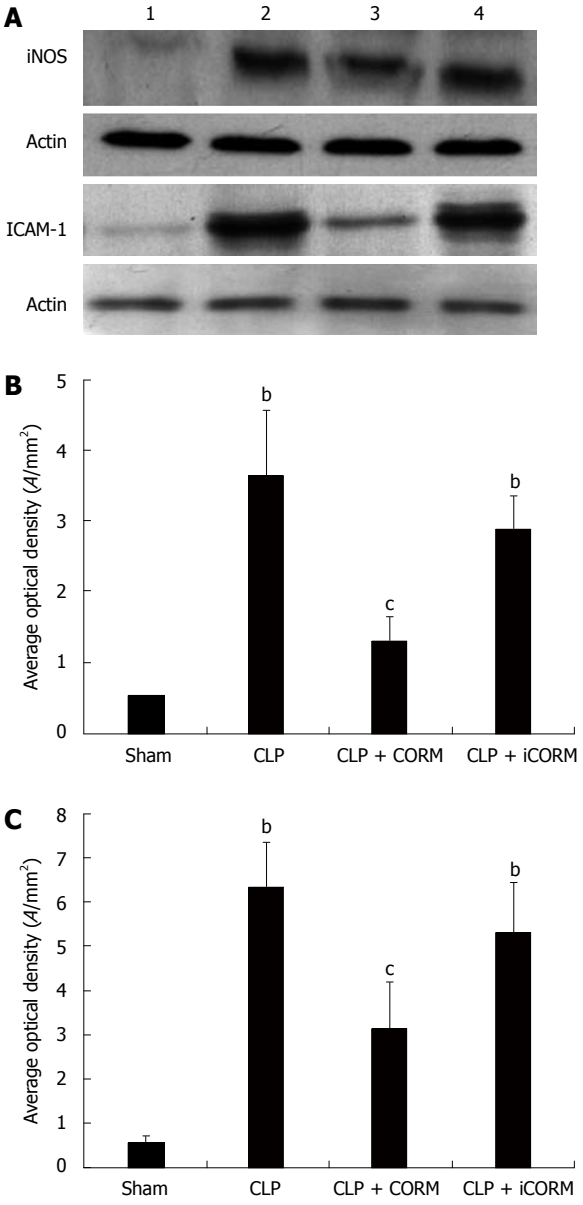


Figure 5 Effects of tricarbonyldichlororuthenium (Ⅱ) dimer carbon monoxide-releasing molecules on protein expression of intercellular adhesion molecule 1 and inducible nitric oxide synthase in the mid-ileum of cecal ligation and perforation-induced mice. A: Representative experiment; B and C: Quantitative results (average optical density, A/mm^2) of three. Mice were challenged with cecal ligation and perforation (CLP) and treated with tricarbonyldichlororuthe-nium (Ⅱ) dimer. Protein expression of intercellular adhesion molecule 1 (ICAM-1) and inducible nitric oxide synthase (iNOS) in the mid-ileum was determined by Western blotting at 24 h after CLP. Lane 1: Sham group; Lane 2: CLP group; Lane 3: CLP + carbon monoxide-releasing molecule (CORM)-2 group; Lane 4: CLP + inactivated-carbon monoxide-releasing molecule (iCORM)-2 group. $^bP < 0.01$ *vs* sham group; $^cP < 0.05$ *vs* CLP group.

MDA levels in CLP groups increased markedly, suggesting that significant increase of oxidative stress occurs at 24 h after experimental manipulation. *In vitro* administration of CORM-2 led to the significantly downregulation of the mean MDA levels in the small intestine of septic mice. This indicated that CORM-2 effectively prevents lipid peroxidation in the small intestine after CLP, consequently decreasing the production of oxidants and reducing the tissue oxidative injury, which contributes to the bowel functional damage to the bowel.

Sepsis alters the concentrations of NO, an inflammatory factor, in plasma and endothelial cells[31]. NO is produced by various types of cells such as macrophages, cardiac myocytes, and vascular smooth muscle and glial cells in response to endotoxin and other inflammatory stimuli. Although it is suggested that NO has a counter-inflammatory activity acting on the cells of the adaptive immune system, in particular, T cells, many studies have well established that NO is indeed a proinflammatory mediator, overproduction of NO plays a major role in the pathophysiology of septic shock, and induction of NOS with consequent excessive NO formation has been proposed as a major factor in pathologic vasodilatation and tissue damage. As an intercellular signaling factor, NO potentially leads to the continuous formation of peroxynitrite. Peroxynitrite damages the organs possibly through lipid peroxidation and/or nitration of cell membrane proteins.

Previously, we demonstrated that thermal injury induced lung neutrophil deposition, lung iNOS expression, and lung damage[9]. We have also shown that NO from iNOS regulated proinflammatory activation, gene expression, and tissue injury in the liver after thermal injury, and CORM-2 inhibited the expression of iNOS in liver tissues, reducing liver injury and tissue PMN infiltration in thermally injured mice[8]. In this study, we measured NO production and expression levels of iNOS in tissue homogenates of the ileum and jejunum of CLP mice following resuscitation to determine whether iNOS was also generated at this site. There was a significant increase of intestinal mucosal iNOS activity within the base of intestinal villi following CLP, while NO production also markedly increased, suggesting that peroxynitrite plays a vital role in CLP-induced intestinal damage. The production of NO and expression of iNOS were significantly inhibited by *in vitro* administration of CORM-2. In parallel, the *in vitro* experiments showed that LPS caused a significant increase of NO production in Caco-2 cells, which was prevented effectively with CORM-2 treatment. These data showed that CORM-2 exhibits, at least partly, an important role in inhibiting iNOS expression, subsequently downregulating the NO production, and attenuating the oxidative stress and tissue damage. NF-κB family members control transcriptional activity of various promoters of proinflammatory cytokines, cellsurface receptors, transcription factors, and adhesion molecules that are involved in intestinal inflammation. Previously, using a thermal injury model in mice, we have shown that CORM-2 plays a pivotal role in inhibition of NF-κB activity in the liver, which subsequently decreases hepatocellular secretion of inflammatory cytokines and burn-related hepatic dysfunction[11]. In this study, NF-κB activity in mid-ileum was elevated by CLP, while it was markedly inhibited by administration of CORM-2 (data not shown). These results show that CORM-2 plays, at least partly, an important role in

inhibition of NF-κB activity in the small intestine.

In conclusion, the data presented in this study suggest a protective role of CORM-2, one of the novel CORMs, in the small intestine of the septic mice. The potential mechanism of this beneficial effect of CORM-2 appears to suppress oxidative stress, and decrease the production of IL-1 and TNF-α. This was accompanied by a decrease of NO production and protein expression of ICAM-1 and iNOS, thus suppressing the tissue damage in the small intestine. However, the therapeutic potential of anti-inflammation or anti-oxidative stress strategies in this setting should be further validated by future studies.

COMMENTS

Background

Sepsis is a complex clinical syndrome resulting from a harmful host inflammatory response to infection. Cecal ligation and puncture (CLP) may induce the activation of an inflammatory cascade, and may lead to sepsis and multiple organ failure. There have been several reports indicating that the inflammatory response syndrome, which contributes to oxidative cell/tissue damage, might frequently be accompanied by leukocyte sequestration in many important organ systems in the body. The increase of production of pro-inflammatory mediators such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) is closely associated with activation of leukocytes and macrophages which were sequestrated in the tissue. During sepsis, the most frequent complications within the gastrointestinal tract are small intestine and mucosal barrier dysfunction. Their previous data indicated that the intestine is one of the most sensitive tissues to ischemia and reperfusion induced by thermal injury.

Research frontiers

Sepsis, which frequently occurs after hemorrhage, trauma, burn, or abdominal surgery, continues to be a challenge in clinic as a leading cause of morbidity and mortality in severely ill patients. Despite therapeutic strategies focus on local host defenses and the inhibition of overwhelming inflammation response, little progress has been achieved. Their previous studies firstly confirmed that carbon monoxide (CO)-releasing molecules (CORM)-released CO attenuated leukocytes sequestration in the liver, lung and small intestine of burned mice by interfering with nuclear factor κB activation, protein expression of intercellular adhesion molecule 1 (ICAM-1) and therefore suppressing endothelial cells pro-adhesive phenotype. However, to date, little is known if CORM-released CO can down-regulate the inflammatory production and oxidative stress in the small intestine in septic mice.

Innovations and breakthroughs

In the present study, the authors found that a protective role of tricarbonyldichlororuthenium (Ⅱ) dimer (CORM-2), one of the novel CORMs, on the small intestine during sepsis. The potential mechanism of this beneficial effect of CORM-2 appears to suppress oxidative stress, decrease the myeloperoxidase activities and production of IL-1 and TNF-α. This was accompanied by a decrease of nitric oxide (NO) production and protein expression of ICAM-1 and inducible NO synthase, and therefore suppress tissue damage in the small intestine. These data indicated that CORM-2 effectively prevents polymorphonuclear neutrophil chemotaxis and infiltration in the tissue after CLP, consequently decreased the production of oxidants, reduced tissue oxidative injury.

Applications

The protective role of CORM-2, one of the CORMs, on the organs during sepsis, and its therapeutic potential in anti-inflammation or anti-oxidative stress will be the novel strategies in the future.

Peer review

The authors report the protective role of CORM-2, one of the CORMs, on the organs during sepsis, and its therapeutic potential in anti-inflammation or anti-oxidative stress. This is a well-written article. The study is interesting and goes along with previous data on modulation of inflammation by CO.

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BRIEF ARTICLE

Intramuscular *vs* intradermal route for hepatitis B booster vaccine in celiac children

Salvatore Leonardi, Andrea Domenico Praticò, Elena Lionetti, Massimo Spina, Giovanna Vitaliti, Mario La Rosa

Salvatore Leonardi, Andrea Domenico Praticò, Elena Lionetti, Massimo Spina, Giovanna Vitaliti, Mario La Rosa, Department of Pediatrics, University of Catania, 95100 Catania, Italy

Author contributions: All authors equally contributed to the realization of the research project and the writing of the paper.

Correspondence to: Salvatore Leonardi, MD, Department of Pediatrics, University of Catania, Via S. Sofia 78, 95100 Catania, Italy. leonardi@unict.it

Telephone: +39-953-782764 Fax: +39-953-782385

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Abstract

AIM: To compare intradermal (ID) and intramuscular (IM) booster doses, which have been used in healthy and high risk subjects, such as healthcare workers, haemodialysis patients, human immunodeficiency virus patients, and renal transplant recipients unresponsive to initial hepatitis B vaccination, in celiac individuals.

METHODS: We conducted our study on 58 celiac patients, vaccinated in the first year of life, whose blood analysis had showed the absence of protective hepatitis B virus (HBV) antibodies. All patients had received the last vaccine injection at least one year before study enrolment and they had been on a gluten free diet for at least 1 year. In all patients we randomly performed an HBV vaccine booster dose by ID or IM route. Thirty celiac patients were revaccinated with recombinant hepatitis B vaccine (Engerix B) 2 μg by the ID route, while 28 celiac patients were revaccinated with Engerix B 10 μg by the IM route. Four weeks after every booster dose, the anti-hepatitis B surface (HBs) antibody titer was measured by an enzyme-linked immune-adsorbent assay. We performed a maximum of three booster doses in patients with no anti-HBs antibodies after the first or the second vaccine dose. The cut off value for a negative anti-HBs antibody titer was 10 IU/L. Patients with values between 10 and 100 IU/L were considered "low responders" while patients with an antibody titer higher than 1000 IU/L were considered "high responders".

RESULTS: No significant difference in age, gender, duration of illness, and years of gluten intake was found between the two groups. We found a high percentage of "responders" after the first booster dose (ID = 76.7%, IM = 78.6%) and a greater increase after the third dose (ID = 90%, IM = 96.4%) of vaccine in both groups. Moreover we found a significantly higher number of high responders (with an anti-HBs antibody titer > 1000 IU/L) in the ID (40%) than in the IM (7.1%) group, and this difference was evident after the first booster dose of vaccination ($P < 0.01$). No side effects were recorded in performing delivery of the vaccine by either the ID or IM route.

CONCLUSION: Our study suggests that both ID and IM routes are effective and safe options to administer a booster dose of HBV vaccine in celiac patients. However the ID route seems to achieve a greater number of high responders and to have a better cost/benefit ratio.

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Key words: Hepatitis B virus; Non responders; Intradermal route; Intramuscular route; Celiac disease

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INTRODUCTION

In literature there are several reports describing a non-responsiveness to hepatitis B vaccine in celiac patients[1-4], although the pathogenic mechanism is still unclear. As a matter of fact it seems that this failed response could be linked to the "major histocompatibility complex" (MHC) and human leucocyte antigen (HLA)-Ⅱ pattern characterizing the disease[5-7], while other studies indicate that gluten intake at the time of vaccination could influence the vaccine-induced immune response[8].

However it is not completely understood whether the unresponsiveness to hepatitis B vaccine in celiac patients is also linked to a weakened immune response in healthy older people or to a physiological loss of humoral immunity with the flow of time[9]. Fisman *et al*[10] published a meta-analysis on the increased risk of unresponsiveness to hepatitis B vaccination in older subjects, finding a low response even in 30-year-old patients.

An important consideration is that the titer of anti-hepatitis B surface (anti-HBs) antibodies that should be considered as cut-off for "non-response" to hepatitis B virus (HBV) vaccine is < 10 IU/L, when the measurement is performed a long time after the vaccination. The responsiveness to hepatitis B vaccine should usually be determined by antibody measurement within 2-6 mo after the third vaccine dose. In those patients with anti-HBs < 10 IU/L a booster vaccine schedule should be proposed, but until now there is no consensus on this kind of management.

In the present study we administered a vaccine booster dose against HBV by the intradermal (ID) or intramuscular (IM) route in celiac patients, whose antibodies levels against the HBV were low after the first regimen of hepatitis B vaccine performed in the first year of life.

The aim of our study was to evaluate the possibility to provide a satisfactory immune response against HBV by these procedures, comparing their efficacy.

MATERIALS AND METHODS

Our study was a prospective, randomized study, conducted on 58 celiac patients (age, mean ± SD, 9.8 ± 6.2 years) of 116 celiac subjects (age, mean ± SD, 10.2 ± 5.7 years) referred to our Pediatric Department, University of Catania, Italy, whose blood analysis showed the absence of protective HBV antibodies (anti-HBs). In all included patients, the diagnosis of celiac disease was made after one year of age, based on clinical signs and standard serological markers (antigliadin IgA and IgG, tissue trans-glutaminase IgA antibody, anti-endomysial antibody) and on typical histological findings on small bowel biopsies (villous atrophy with crypt hyperplasia and increased intraepithelial lymphocytes).

All patients received the anti-HBV vaccine at 3, 5 and 11 mo of age by IM injection on the front-lateral area of their quadriceps muscle (10 μg of Engerix B, GlaxoSmith and Kline, Belgium), as planned by the Italian standard vaccination schedule.

All patients had received the last vaccine injection at least one year before the study enrolment and they had been on a gluten free diet for at least 1 year. None of the patients had ever been affected by HBV infection.

In all patients we randomly performed an HBV vaccine booster dose by the ID or IM route. Thirty celiac patients were revaccinated by the ID route with a 2 μg dose of recombinant hepatitis B vaccine (Engerix B) administered on the flexor surface of the forearm, using a 1 mL syringe and 26-gauge needle. In all the patients a visible skin weal was noticed as evidence of the ID inoculation.

Twenty-eight celiac patients were revaccinated by the IM route with a 10 μg dose of Engerix B administered in the lateral region of their deltoid muscle, using a 5 mL syringe and 26-gauge needle.

Four weeks after every booster dose, the anti-HBs antibody titer was measured by enzyme-linked immune-adsorbent assay (hepanostica anti-HBs, bioMeriuex, Netherlands). We performed a maximum of three booster doses in patients with no anti-HBs antibodies after the first or the second vaccine dose. The cut off value for a negative anti-HBs antibody titer was 10 mIU/mL. Patients with values between 10 and 100 IU/L were considered "low responders" while patients with an antibody titer higher than 1000 IU/L were considered "high responders"[11].

Statistical analysis

The Mann-Withney *U*-test was performed to compare age, duration of illness, years of gluten intake and HBs antibody titer between the two groups of patients. The Fisher exact test was used to compare the gender, the number of non responders, low responders and high responders between the ID and IM groups. *P* value < 0.05 was considered statistically significant.

RESULTS

The main features of the two groups of patients are reported in Table 1. No significant difference of age, gender, duration of illness, and years of gluten intake was found between the two groups.

The number and the percentage of responders to ID and IM hepatitis B vaccination after every dose injection are reported in Table 2, together with the mean and SD of the anti-HBs titer in the two groups after the first and the third booster.

Both groups of patients showed a similar percentage of responders after the first dose of vaccine (ID = 76.7%, IM = 78.6%) and a major increase after the third dose (ID = 90%, IM = 96.4%). However, we did not find any statistically significant difference between the two groups. We found no statistically significant difference in anti-HBs titer between the two groups, after the first and the third doses.

Finally we found a significantly higher number of high responders (with an anti-HBs antibody titer > 1000 IU/L) in the ID (40%) than in the IM (7.1%) group, and this difference was evident after the first booster dose of

Table 1 Comparison of age, gender, duration of illness and gluten intake in patients receiving vaccine booster by the intradermal or intramuscular route

	Intradermal	Intramuscular	*P* value
Age (yr)	10.45 ± 6.7[1]	9.3 ± 5.9[1]	NS[2]
Gender (male/female)	10/20	9/19	NS[3]
Duration of illness (yr)	6.15 ± 4.1[1]	7.5 ± 4.6[1]	NS[2]
Gluten Intake (yr)	5.3 ± 3.7[1]	4.6 ± 3.2[1]	NS[2]

NS: Not significant. [1]mean ± SD; [2]Mann-Whitney *U*-test; [3]Fisher exact test.

Table 2 Number and percentage of responders to the different booster doses and comparison of anti-hepatitis B surface titer after the first and the third doses

Booster doses	Intradermal		Intramuscular		*P* value
	Responders	Anti-HBs titer	Responders	Anti-HBs titer	
First	23/30 (76.7)	433.35 ± 476.76	22/28 (78.6)	280.4 ± 328.97	NS[1]
Second	4/30 (13.3)		5/28 (17.8)		NS[2]
Third	0/30 (0)	459 ± 455.87	0/28 (0)	294 ± 320.8	NS[1]
Total	27/30 (90)		27/28 (96.4)		NS[2]

Data are presented as mean ± SD or *n* (%). [1]Fisher exact test (intradermal *vs* intramuscular responders) and Mann-Whitney *U*-test (antibodies titer comparison); [2]Fisher exact test (intradermal *vs* intramuscular responders). NS: Not significant; HBs: Hepatitis B surface.

vaccination (Figure 1). No side effects were recorded in performing both ID and IM injections.

DISCUSSION

Literature data describe that 4%-10% of healthy, immune competent individuals fail to elicit protective levels of antibodies to recombinant HBs antigen after completing the standard hepatitis B vaccination schedule[12]. Even though the pathogenic mechanism leading to a failed response to hepatitis B vaccine is still unknown, there are several hypotheses trying to explain this link. Recently Zingone *et al*[8] reported a possible association with gluten intake at the time of vaccination that may influence the vaccine-induced immune response. Nevertheless the most likely hypothesis is related to a specific pattern of MHC[13] and HLA-Ⅱ antigens linked to the disease. As a matter of fact, homozygosis for HLA-B8, DR3 and DQ2 alleles was found to have a significantly higher incidence in hepatitis B vaccine non responders[5-7].

This HLA-DQ2 haplotype is present in 90%-95%[14,15] of celiac patients and it seems to explain the relationship between the disease and the non-responsiveness to hepatitis B vaccine. Thus, in celiac non responders a re-vaccination should be recommended because of the worldwide spread of the disease.

Nowadays, there is no consensus on the management of celiac patients with anti-HBs antibody levels < 10 IU/L after the IM vaccine. In healthy people a common practice is to administer a higher dose of HBV re-

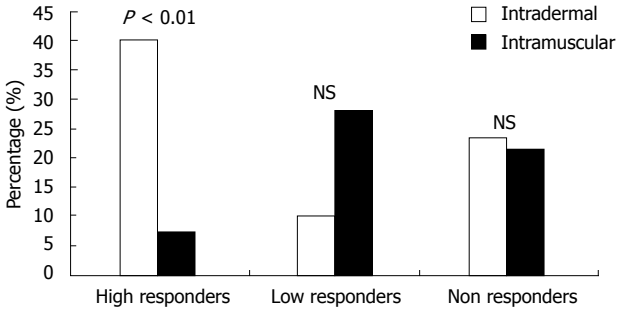


Figure 1 Percentage of high responders, low responders and non responders after the first booster dose. *P* value was calculated by Fisher exact test. NS: Not significant.

combinant vaccine (HBRV) or a second course of three doses of IM recombinant vaccine (IMRV)[16], but it does not seem to be successful. In fact it has been reported that more than 50% of non responders are not able to acquire an protective anti-HBs titer with at least two additional IMRV booster doses in the primary course[17,18].

In human immunodeficiency virus patients, repeated vaccination is commonly considered as a first satisfactory strategy[19]. Some investigators have even increased the dose of hepatitis B vaccine with varying success[20,21] or have used a double dose of a combined hepatitis A and B vaccine[22].

The United States Center for Disease Control and Prevention recommends the administration of an additional series of three doses of IM vaccine in chronic hemodialysis patients[23]. For those non-responders after two series (six doses of vaccine in total), there is no data to support the use of additional doses to induce an immune response.

Another approach is to administer HBRV vaccine by the ID route. In fact a recent meta-analysis by Fabrizi *et al*[24] concluded that the ID route is associated with higher anti-HBs antibody levels, although this is not sustained over time. Recently in a pilot study we found an effective response after ID administration of HBRV in celiac patients too[25].

At present this is the first study comparing the ID and IM routes in these patients. In our study we found a high percentage of response after the first dose of vaccination in both groups (ID 76.7% *vs* IM 78.6%) and a higher response after the third booster dose (ID 90% *vs* IM 96.4%). Moreover, the percentage of responders in both groups after the three doses of vaccine was similar to those found in vaccinated healthy people[12].

Our data confirms that both routes are effective to perform a booster strategy in celiac patients with low anti-HBs antibodies, as 90% of ID patients and 96.4% of IM subjects showed a protective anti-HBs titer after the third booster dose. However the ID route seems to produce a significantly higher percentage (40%) of high responders (anti-HBs > 1000 IU/L) than the IM route (7.1%).

In our opinion, this result may have an important clinical significance, because a protective anti-HBs titer

may persist to 64% after 10 years in normal children if there is a high value of anti-HBs antibody titer at the end of the initial schedule[9].

However, whether the ID route is a better strategy than IM hepatitis B vaccine still remains an open question. In fact several studies in high-risk groups[26-28] showed that low dose ID injections resulted in long term sero-protection in a large number of subjects non responsive to IM vaccination. However, a recent meta-analysis of 757 adults by Sangaré *et al*[29] demonstrated that ID hepatitis B vaccination was less effective to achieve sero-protection than IM vaccination.

Recently a randomized study on ID *vs* IM hepatitis B vaccination in human immunodeficiency virus-infected children, without severe immunosuppression, confirmed this issue[30]. In particular, in the study by Medeiros *et al*[31] on hemodialysis patients, the percentage of responders was very low (13.3%).

Our data seem to suggest that the use of the ID route for the booster dose of hepatitis B vaccine in celiac patients is a better option to obtain an higher titer of antibodies against HBV. Moreover the ID route allows a better cost/efficacy ratio, because of the cost reduction exceeding 50% (2 μg per dose) compared with a standard IM vaccine regimen (10 μg per dose)[32]. In conclusion, it is important to highlight that the ID route could represent an efficacious and cost-saving option for difficult-to-vaccinate and high-risk patients, as reported in other studies[33-35] and also for the observed 4%-10% of healthy people who normally fail to respond to the standard HBV vaccination regimen[36].

COMMENTS

Background

The hepatitis B vaccine is a mandatory vaccine for all children. Nevertheless around 4%-10% of healthy subjects and a higher percentage of patients affected by celiac disease do not respond to the standard cycle of vaccination provided by the Ministry of Health. As a matter of fact they show an antibody titer less than 10 IU/L after the standard vaccination doses. This population could be considered at risk for potential exposure to hepatitis B virus (HBV) infection. Thus, it is proposed that the revaccination of "non-responders" at the first cycle of scheduled HBV vaccination, by booster doses, could improve HBV antibody titer and this study compared the efficacy of intramuscular (IM) boosters *vs* intradermal (ID) vaccination.

Research frontiers

The ID route of vaccination is an effective way to vaccinate people, it is safe and it seems to be easier to practice than the IM route. This way should be considered in "non-responder" celiac patients, not only for the high response to vaccination, but also because it allows a better cost/efficacy ratio, with a cost reduction exceeding 50% compared with an IM vaccine regimen. This cost decrease is linked to the lower dosage of vaccine used in the ID route (2 μg per dose) than in the IM route (10 μg per dose).

Innovations and breakthroughs

The problems of responsiveness to vaccinations such as anti HBV vaccine have been widely studied, and researchers have demonstrated how this lack of response could be linked to genetic and environmental factors. Actually there are few studies that respond to the question on how to solve this non-responsiveness to HBV vaccine. Some studies found a good strategy was re-vaccination by the IM route, others demonstrated a better outcome with the ID route. Moreover, other researchers find the practice of applying booster doses to "non-responders" not useful, as they conserve their intra-cellular immunity. Nevertheless, as the problem has widely interested the scientific community in these last years, it could be useful to establish a common behavior to solve this question. For this reason, studies on new vaccination schedules could be useful and this study is the first one that compares two vaccination routes in order to better understand the way the research should address its efforts.

Applications

The study results suggest that ID route of HBV vaccination is a potential therapeutic strategy that could be used in improving responsiveness to HBV vaccine and in preventing a potential infection in a "non-responders" population.

Peer review

This is a good prospective, randomized study in which the authors analyzed the efficacy of ID and IM routes in HBV vaccinated children to improve their responsiveness to standard vaccination. The results are interesting and suggest that the ID route of booster vaccination is not only as effective as the IM one, but it is also less expensive, so it could easily be considered as a successful strategy for booster doses of HBV vaccine in "non-responder" patients.

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BRIEF ARTICLE

Prognostic factors for recurrence of gastrointestinal bleeding due to Dieulafoy's lesion

Yuliana Jamanca-Poma, Antonio Velasco-Guardado, Concepción Piñero-Pérez, Renzo Calderón-Begazo, Josue Umaña-Mejía, Fernando Geijo-Martínez, Antonio Rodríguez-Pérez

Yuliana Jamanca-Poma, Antonio Velasco-Guardado, Concepción Piñero-Pérez, Renzo Calderón-Begazo, Josue Umaña-Mejía, Fernando Geijo-Martínez, Antonio Rodríguez-Pérez, Department of Digestive System, University Hospital of Salamanca, 37007 Salamanca, Spain
Author contributions: Jamanca-Poma Y, Piñero-Pérez C and Calderón-Begazo R collected data; Velasco-Guardado A statistically analyzed data; Jamanca-Poma Y and Umaña-Mejía J wrote the paper; Geijo-Martínez F and Rodríguez-Pérez A reviewed the paper.
Correspondence to: Dr. Antonio Velasco-Guardado, Department of Digestive System, University Hospital of Salamanca, 37007 Salamanca, Spain. antoniovelascog@usal.es
Telephone: +34-92-3291400 Fax: +34-92-3291131
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Abstract

AIM: To analyze the effectiveness of the endoscopic therapy and to identify prognostic factors for recurrent bleeding.

METHODS: Retrospective study of patients with gastrointestinal bleeding secondary to Dieulafoy's lesion (DL) from 2005 to 2011. We analyzed the demographic characteristics of the patients, risk factors for gastrointestinal bleeding, endoscopic findings, characteristics of the endoscopic treatment, and the recurrence of bleeding. We included cases in which endoscopy described a lesion compatible with Dieulafoy. We excluded patients who had potentially bleeding lesions such as angiodysplasia in other areas or had undergone other gastrointestinal endoscopic procedures.

RESULTS: Twenty-nine patients with DL were identified. Most of them were men with an average age of 71.5 years. Fifty-five percent of the patients received antiaggregatory or anticoagulant therapy. The most common location for DL was the stomach (51.7%). The main type of bleeding was oozing in 65.5% of cases. In 27.6% of cases, there was arterial (spurting) bleeding, and 6.9% of the patients presented with an adherent clot. A single endoscopic treatment was applied to nine patients (31%); eight of them with adrenaline and one with argon, while 69% of the patients received combined treatment. Six patients (20.7%) presented with recurrent bleeding at a median of 4 d after endoscopy (interquartile range = 97.75). Within these six patients, the new endoscopic treatment obtained a therapeutic success of 100%. The presence of arterial bleeding at endoscopy was associated with a higher recurrence rate for bleeding (50% *vs* 33.3% for other type of bleeding) [P = 0.024, odds ratio (OR) = 8.5, 95% CI = 1.13-63.87]. The use of combined endoscopic treatment prevented the recurrence of bleeding (10% *vs* 44.4% of single treatment) (P = 0.034, OR = 0.14, 95% CI = 0.19-0.99).

CONCLUSION: Endoscopic treatment of DL is safe and effective. Adrenaline monotherapy and arterial (spurting) bleeding are associated with a high rate of bleeding recurrence.

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Key words: Dieulafoy's lesion; Gastrointestinal bleeding; Hemorrhage; Recurrent bleeding; Endoscopic treatment

Peer reviewers: Anastasios Koulaouzidis, MD, MRCP, Day Case and Endoscopy Unit, Centre of Liver and Digestive Disorders, Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh, Scottish EH16 4SA, United Kingdom; Grigoriy E Gurvits, MD, Department of Gastroenterology, St. Vincent's Hospital and Medical Center, New York Medical College, 153 West 11th Street, Smith 2, New York, NY 10011, United States

Jamanca-Poma Y, Velasco-Guardado A, Piñero-Pérez C, Calderón-Begazo R, Umaña-Mejía J, Geijo-Martínez F, Rodríguez-

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INTRODUCTION

Dieulafoy's lesion (DL) was first described in 1884 by Gallarden[1-3], and characterized in 1898 by the French surgeon George Dieulafoy, who described its characteristics in three patients and gave it its name. It is a rare but important cause of gastrointestinal bleeding. Up to 6% of the cases of nonvaricose bleeding in the upper gastrointestinal tract[4,5] and 1%-2% of all gastrointestinal hemorrhages are caused by DL[6,7]. In spite of this, its mortality rates are similar to those of other causes for gastrointestinal bleeding[8]. This lesion is defined by the presence of an abnormally thick arteriole (1-3 mm in diameter, 10 times higher than the diameter of submucosal capillaries at that level) that maintains the caliber of the feeding vessel when it reaches the mucous membrane[3,9]. Histologically, it is characterized by subintimal fibrosis of the artery and a lack of inflammation at the edge of the mucosal defect, which sets it apart from peptic ulcers. The exact mechanism that triggers the bleeding is unknown. Some authors have speculated on the combined effect of atrophy and ischemia of the mucosa[2]. It is believed that this thick vessel applies pressure to the mucosa and leads to a small erosion and rupture of the vessel towards the lumen. Clinically, it appears with intermittent and painless gastrointestinal bleeding[10,11]. The most common location is the stomach[6,8]; specifically the lesser curvature[1,8,12]. In 80%-95% of these cases it is located within 6 cm of the gastroesophageal junction, because the arterial flow in this area emerges directly from the left gastric artery[2,13,14]. Approximately one third of the lesions are located outside the stomach, most of them in the duodenum[15], followed by the colon[3,16]. They have also been described in the esophagus, jejunum and ileum[13,17], rectum[18,19] and anal canal[20]. There have also been some cases in which the lesion was outside the gastrointestinal tract (bronchial tubes)[21].

The diagnostic procedure of choice is endoscopy, which is effective in > 90% of patients[7,8]. The endoscopic diagnosis may be difficult, especially during the first episode, due to the size of the lesion and the intermittent nature of the hemorrhage. For this reason, up to 6% of patients require three or more endoscopies in order to reach a diagnosis[8]. Endoscopic ultrasound has been highlighted as a useful technological resource that can support the diagnosis of this condition, because it can locate the submucosal vessel with great accuracy[22].

Traditionally, the treatment of DL was surgical. However, with the development of endoscopic hemostasis techniques, the need for surgery has been reduced, and the mortality rates have decreased from 80% to 8.6%[23]. Therefore, the current treatment of choice in accessible lesions is endoscopy[13], with a success rate of > 90%[7,24-26] and low rates of recurrence and complications[27-33]. The endoscopic hemostasis procedures are classified into three groups: (1) thermal: electrocoagulation, heater probe and argon plasma coagulation; (2) local injection of substances, such as adrenaline or sclerosing solutions; and (3) mechanical: hemostatic clips and bands[6,7,12].

All these procedures achieve high rates of primary hemostasis, with low rates of recurrence (generally < 10%)[27-33]. However, in the clinical field, the choice of a procedure depends on the experience and the decision of the endoscopist.

The objectives of our study were to assess the effectiveness of endoscopic treatment of DL, and to identify potential prognostic factors for recurrence of bleeding in patients with this kind of lesion.

MATERIALS AND METHODS

Patients

This was a retrospective study of patients who were admitted to the University Hospital of Salamanca with a diagnosis of gastrointestinal bleeding caused by DL between 2005 and 2011. We collected data regarding clinical presentation of the disease, diagnosis, and effectiveness of medical and endoscopic treatment of gastrointestinal bleeding caused by DL. We included cases in which endoscopy described a lesion compatible with Dieulafoy. We excluded patients who had potentially bleeding lesions such as angiodysplasia in other areas or had undergone other gastrointestinal endoscopic procedures.

Descriptive analysis

In the descriptive analysis of the sample, we used percentages for the qualitative variables and measures of central tendency (mean and/or median when there was a large asymmetry or dispersion) and of dispersion (SD) for the quantitative variables.

Statistical analysis

We used the SPSS Version 15.0 (Chicago, IL, United States) for the analysis. The dichotomous variables were analyzed with the χ^2 test on contingency tables in order to compare the distribution of categorical variables, and we calculated the odds ratios (ORs) and 95% CIs. The analysis of variance test was used in the analysis of continuous and categorical variables. The multivariate analysis was done using binary logistic regression. Statistical significance was established at $P < 0.05$.

RESULTS

Out of the 2582 patients admitted in the endoscopy unit with upper gastrointestinal bleeding, we identified 29 with DL (1.12%). In this group, 21 (72.4%) patients were

Table 1 Prognostic factors analyzed in cases of bleeding recurrence caused by Dieulafoy's lesion *n* (%)

Factor	Bleeding recurrence	No bleeding recurrence	*P* value
Male patients	4 (66.7)	17 (73.9)	0.54
Arterial hypertension	5 (83.3)	13 (56.5)	0.23
Ischemic heart disease	3 (33.3)	6 (20.1)	0.54
Valvular heart disease	1 (16.7)	5 (21.7)	0.63
Renal failure	1 (16.7)	-	-
Anticoagulant or antiaggregant	3 (50)	13 (56.5)	0.56
Antiaggregant	6 (100)	7 (30.4)	0.62
Anticoagulant	1 (16.7)	11 (47.8)	0.18
Location in the stomach	3 (50)	12 (52.2)	0.63
Mean systolic arterial pressure (mmHg)	107.3	112	0.62
Mean heart rate (beats/min)	83	85.7	0.77
Urea (mg/dL)	76.8	84.3	0.66
Hemoglobin (mg/dL)	8.76	8.75	0.98
Need of transfusion	6 (100)	20 (87)	0.48
PPI perfusion	2 (33.3)	11 (47.8)	0.43
Arterial bleeding type	4 (66.7)	4 (19)	0.02
Combined endoscopic treatment	2 (33.3)	18 (78.3)	0.03

PPI: Proton-pump inhibitor.

men with an average age of 71.5 ± 14.5 years. Their personal records showed arterial hypertension (62.1%), ischemic heart disease (27.6%), valvular heart disease (20.7%), and chronic liver disease (3.4%). Sixteen patients (55.2%) were receiving antiaggregatory or anticoagulant therapy, and five (17.2%) a combination of both therapies.

Among the patients treated with antiaggregatory agents, five (55.5%) were being treated with acetylsalicylic acid (ASA), and the remainder with clopidogrel. Among the anticoagulant patients, 11 (91.6%) were being treated with acenocoumarol, and one patient was being treated with low molecular weight heparin. No patient was receiving triple therapy (ASA/clopidogrel/acenocoumarol). Only three (10.3%) of the patients were being treated with a proton-pump inhibitor (PPI) before the bleeding episode.

Symptoms of clinical instability were present in 10 (34.5%) patients (systolic arterial pressure < 100 mmHg and/or heart rate > 100 beats/min) and the average hemoglobin levels before gastroscopy were 8.6 ± 2.48 mg/dL.

The most common location for DL was the stomach (51.7%), followed by duodenum (31%) and esophagus (13.8%). In one patient, the lesion was located on the colon. The main type of bleeding was oozing, in 65.5% of the cases. In 27.6% of the cases there was arterial (spurting) bleeding (Figure 1), and 6.9% of the patients presented with an adherent clot. A single endoscopic treatment was applied to nine patients (31%); eight of them with adrenaline and one with argon, while 69% of the patients received combined treatment (several different combinations with clip, adrenaline, sclerosing therapy and argon). Hemostatic bands were not used in any of the cases. All medical treatments were initiated before endoscopy, 16 patients (55.2%) received continuous intravenous perfusion of PPI with an average dose of 156.92 ± 62.1 mg/24 h for an average period of 3 ± 1.82 d, and 13 (44.8%) patients received treatment with oral PPI, with an average dose of 57.5 ± 34.93 mg/24 h. Most of the patients (n = 26) required red blood cell transfusion, with a median value of 2 U concentrate per patient [interquartile range (IQR) = 2].

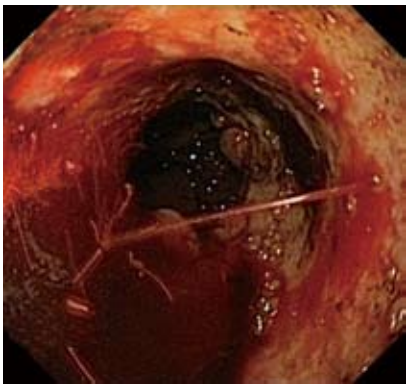


Figure 1 Dieulafoy's lesion located on the esophagus with arterial (spurting) bleeding.

Bleeding reappeared in six patients (20.7%), at a median 4 d after endoscopy (IQR = 97.75). In this group of patients, the second endoscopic treatment achieved a therapeutic success rate of 100%. The recurrence of bleeding was not associated with age, sex, personal record, previous medical treatment (including antiaggregants and/or anticoagulants), the seriousness of the hemorrhage or its location, or the medical treatment used (Table 1). However, the presence of arterial (spurting) bleeding at endoscopy was associated with a higher rate of bleeding recurrence (50% *vs* 33.3% for other types of bleeding) (P = 0.024, OR = 8.5, 95% CI = 1.13-63.87). Multivariate analysis adjusted for age, sex, type of antiaggregant, type of anticoagulation and single or combined endoscopic treatment, confirmed the association (P = 0.039). The use of combined endoscopic treatment prevented recurrence of bleeding (10% *vs* 44.4% with monotherapy) (P = 0.034, OR = 0.14, 95% CI = 0.19-0.99), and multivariate analysis confirmed this association (P = 0.049). Two patients died; one due to postoperative septic shock and the other to complications of previous heart disease.

DISCUSSION

In our hospital, the prevalence of gastrointestinal bleeding due to DL was 1.12%. This percentage was similar to that in other studies[23,31,34]. The demographic characteristics of our patients were similar to those in other studies; we found a higher prevalence of the condition in men, compared to women, with a ratio of 3:1, and an average age of 71.5 years. DL usually affects patients with associated comorbidity[25], such as cardiovascular disease, arterial hypertension or renal failure. Some authors have proposed that these conditions alter the normal process of angiogenesis and trigger the formation of aberrant ves-

sels with a constant caliber that increase the incidence of DL[35]. In our study, most of the patients presented with comorbidity; mainly hypertension (62.1%) and ischemic heart disease (27.6%). Similarly, some researchers have proposed a causal relation with the use of nonsteroidal anti-inflammatory drugs and aspirin, and they argue that the erosive gastritis and the subsequent necrosis of the vascular wall induced by these drugs may lead to rupture of the submucosal vessels, although there is little evidence in the literature that supports this relationship[1,2]. In our study, although a high percentage of the patients received antiaggregant therapy, only three patients (10%) received some additional PPI.

Gastrointestinal bleeding caused by DL can be serious. In our study, 10 patients (34.5%) presented with symptoms of hemodynamic instability, and 27 (93.1%) showed active bleeding at the moment of endoscopic exploration. Two patients died; one due to postoperative complications (septic shock) and the other to complications of previous heart disease.

Most of the studies have shown that the most common location of DL is the stomach. In our series this was also the most common location, followed by the duodenum and the esophagus, plus one case in which the lesion was in the colon and two cases in which it was located on the gastric anastomosis caused by gastrectomy.

The first endoscopy was diagnostic in 93% of cases. Early exploration seems to increase sensitivity due to the intermittent nature of bleeding caused by DL[31] but we did not study this factor.

The success rate of endoscopic treatment in our study was high, similar to that reported in other studies[23,36,37]. Primary hemostasis was achieved in 89% of the cases ($n = 26$).

The endoscopic methods used were: injection (adrenaline, aethoxysklerol), thermal techniques (argon), and mechanical techniques (clip). In most of the cases, combined endoscopic treatment was applied (69%), and the technique was chosen according to the criteria of the endoscopist. The most commonly used technique was a combination of adrenaline injection and a sclerosing agent. Recurrent bleeding was found in six cases (20.7%), compared with other studies that have described rates of 9%-40%[23]. The recurrence of bleeding was associated with monotherapy with adrenaline and endoscopic findings of arterial (spurting) bleeding. Although there is no consensus on the endoscopic treatment of choice, several studies have compared the effectiveness of different techniques. Adrenaline injection is the most commonly used method due to its availability, low cost and safety. Some studies have shown a higher effectiveness of mechanical methods compared with injection techniques. In recent years, several studies have proven that the hemoclip[35] and endoscopic ligature improve the rates of primary hemostasis and reduce the need for subsequent endoscopy[38,39]. Theoretically, the mechanical hemostasis leads to a smaller lesion of the surrounding tissue than thermal therapy or injection techniques, which suggests that these procedures could be a first-line approach for the management of DL[33,36,40]. Another study has shown better results for thermocoagulation *vs* injection of a single substance[37]. A recent study has concluded that combined therapy with hemoclip and aethoxysklerol injection is the most effective[41], while other studies have shown that sclerosis with adrenaline was associated with a higher risk of bleeding recurrence[29,42]. Similar to endoscopic treatment of bleeding due to peptic ulcer, a lower rate of bleeding recurrence has been described with the use of combined endoscopic treatment compared with monotherapy, as could be seen in our study.

With regard to the limitations of our study, we must first mention the small size of the sample, and the retrospective design. The low number of prospective studies may be due to the low incidence of DL.

In conclusion, endoscopic treatment of DL is effective. Treatment with adrenaline monotherapy, and arterial (spurting) bleeding, are associated with a higher rate of bleeding recurrence. For this reason, combined treatment is recommended.

COMMENTS

Background

The Dieulafoy's lesion (DL) is a rare condition that causes gastrointestinal bleeding and treatment is difficult. It is a small aberrant vessel and the diagnosis is only obtained after surgery and pathological analysis. Recurrent bleeding is common and we have many endoscopic therapeutic options.

Research frontiers

Because it is very rare, there have been few prospective studies with few patients to analyze the characteristics of the bleeding, factors associated with recurrence, and treatment success. Although there is no consensus on the endoscopic treatment of choice, several studies have compared the effectiveness of different techniques.

Innovations and breakthroughs

The authors showed that combined treatment with two endoscopic techniques was more effective in controlling bleeding than adrenaline monotherapy, as occurs for gastrointestinal ulcer bleeding. Furthermore, it is important to consider the type of presentation of bleeding. Arterial (spurting) bleeding is associated with a higher rate of recurrence.

Applications

This study suggests that when a DL with active bleeding is present, combination therapy should be performed with at least two endoscopic techniques, especially if the bleeding is arterial.

Peer review

The authors have retrospectively analyzed patients with bleeding from DL in whom endoscopic therapy in the form of single or combination treatment was applied. The authors have then tried to establish factors responsible for rebleeding. Interesting paper on an interesting topic.

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BRIEF ARTICLE

Incidence of human papilloma virus in esophageal squamous cell carcinoma in patients from the Lublin region

Andrzej Dąbrowski, Wojciech Kwaśniewski, Tomasz Skoczylas, Wiesława Bednarek, Dorota Kuźma, Anna Goździcka-Józefiak

Andrzej Dąbrowski, Tomasz Skoczylas, Second Department of General and Gastrointestinal Surgery, Oncological Surgery of the Alimentary Tract, Medical University of Lublin, 20-081 Lublin, Poland
Wojciech Kwaśniewski, Wiesława Bednarek, Department of Gynecological Oncology and Gynecology, Medical University of Lublin, 20-081 Lublin, Poland
Dorota Kuźma, Anna Goździcka-Józefiak, Department of Molecular Virology, Institute of Experimental Biology, Adam Mickiewicz University, 61-614 Poznań, Poland
Author contributions: Dąbrowski A designed the research; Dąbrowski A, Skoczylas T, Kuźma D, Goździcka-Józefiak A, Kwaśniewski W and Bednarek W performed the research; Dąbrowski A, Kwaśniewski W and Skoczylas T analyzed the data, wrote, reviewed and accepted the paper.
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Correspondence to: Dr. Tomasz Skoczylas, Second Department of General and Gastrointestinal Surgery, Oncological Surgery of the Alimentary Tract, Medical University of Lublin, Staszica 16, 20-081 Lublin, Poland. tomskocz@yahoo.com
Telephone: +48-81-5328810 Fax: +48-81-5328810
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Abstract

AIM: To assess the prevalence of human papilloma virus (HPV) in esophageal squamous cell carcinoma (ESCC) in the south-eastern region of Poland.

METHODS: The study population consisted of 56 ESCC patients and 35 controls. The controls were patients referred to our department due to other non-esophageal and non-oncological disorders with no gross or microscopic esophageal pathology as confirmed by endoscopy and histopathology. In the ESCC patients, samples were taken from normal mucosa (56 mucosa samples) and from the tumor (56 tumor samples). Tissue samples from the controls were taken from normal mucosa of the middle esophagus (35 control samples). Quantitative determination of DNA was carried out using a spectrophotometric method. Genomic DNA was isolated using the QIAamp DNA Midi Kit. HPV infection was identified following PCR amplification of the *HPV* gene sequence, using primers MY09 and MY11 complementary to the genome sequence of at least 33 types of HPV. The sequencing results were computationally analyzed using the basic local alignment search tool database.

RESULTS: In tumor samples, HPV DNA was identified in 28 of 56 patients (50%). High risk HPV phenotypes (16 or/and 18) were found in 5 of 56 patients (8.9%), low risk in 19 of 56 patients (33.9%) and other types of HPV (37, 81, 97, CP6108) in 4 of 56 patients (7.1%). In mucosa samples, HPV DNA was isolated in 21 of 56 patients (37.5%). High risk HPV DNA was confirmed in 3 of 56 patients (5.3%), low risk HPV DNA in 12 of 56 patients (21.4%), and other types of HPV in 6 of 56 patients (10.7%). In control samples, HPV DNA was identified in 4 of 35 patients (11.4%) with no high risk HPV. The occurrence of HPV in ESCC patients was significantly higher than in the controls [28 of 56 (50%) *vs* 4 of 35 (11.4%), $P < 0.001$]. In esophageal cancer patients, both in tumor and mucosa samples, the predominant HPV phenotypes were low risk HPV, isolated 4 times more frequently than high risk phenotypes [19 of 56 (33.9%) *vs* 5 of 56 (8.9%), $P < 0.001$]. A higher prevalence of HPV was identified in female patients (71.4% *vs* 46.9%). Accordingly, the high risk phenotypes were isolated more frequently in female patients and this difference reached statistical significance [3 of 7 (42.9%) *vs* 2 of 49 (4.1%), $P < 0.05$]. Of the pathological characteristics, only an infiltrative pattern of macroscopic tumor type significantly correlated with the presence of HPV DNA in ESCC samples [20 of 27 (74.1%) *vs* 8 of 29 (27.6%) for ulcerative or protruding macroscopic type, $P < 0.05$]. The occurrence of total HPV DNA and both HPV high or low risk phenotypes did not significantly differ with regard to particu-

lar grades of cellular differentiation, phases in depth of tumor infiltration, grades of nodal involvement and stages of tumor progression.

CONCLUSION: Low risk HPV phenotypes could be one of the co-activators or/and co-carcinogens in complex, progressive, multifactorial and multistep esophageal carcinogenesis.

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Key words: Human papilloma virus; Low risk phenotypes; High risk phenotypes; Esophageal cancer; Squamous cell carcinoma; Carcinogenesis

Peer reviewers: Satoshi Osawa, MD, MD, PhD, Assistant Professor, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan; Yukie Sato Kuwabara, MSc, PhD, Centro Internacional de Ensino e Pesquisa, Hospital AC Camargo, São Paulo, SP 01508-010, Brazil

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INTRODUCTION

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related death worldwide[1-3]. Overall survival rates range between 5% and 16%[2]. Thus, esophageal cancer poses a considerable medical and public health challenge in many parts of the world. Although there is a marked tendency for increasing incidence rates of esophageal adenocarcinoma in industrialized Western countries, the world leading pathological type of esophageal cancer is still squamous cell carcinoma[2]. Marked geographic, cultural and ethnic variations suggest the importance of environmental influences in the development of this condition. In China, India and Central Asia (the Esophageal Cancer Belt), squamous cell carcinoma still predominates[2]. Significantly high incidence rates have been reported in France, South Africa, Chile, Brazil and the north-eastern Himalayan region[1-3]. The North American and European countries have been found to be low-risk areas[1-3]. The highest incidence and mortality have been reported in Kazakh populations, especially in Xinjiang, China[1-3]. In Europe, the incidence of esophageal cancer is increasing and seems to parallel the decrease in gastric cancer incidence[1-3]. Poland is a low-incidence country with the reported age-standardized annual incidence of 4.5 and 0.7/100.0, for men and women, respectively[4].

In Western countries, esophageal carcinomas with squamous cell differentiation typically arise after many years of tobacco and alcohol abuse[5]. Other causes include chronic mucosal injury due to hot beverages and malnutrition[5]. An etiologic role of different microorganisms such as fungi, bacteria and viruses has also been proposed for the pathogenesis of esophageal squamous cell cancer (ESCC)[5,6]. Of these microorganisms, human papilloma virus (HPV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) have been investigated[5,6]. The role of HPV in the etiology of ESCC has been debated for the past 30 years[7]. The prevalence of HPV in esophageal cancer seems to be higher in areas with a high incidence of ESCC, such as China and South Africa. In 1982, Syrjanen *et al*[7] found that HPV infection caused pathological lesions in esophageal cancer specimens. The prevalence of HPV in ESCC in subsequent studies has shown high variability ranging from 0% to 67%[8-12]. The issue of HPV incidence in ESCC patients in the Polish population has not yet been addressed. In this study we evaluated the prevalence of HPV DNA in patients treated in a regional specialized center for esophageal cancer surgery in Poland, where squamous cell type differentiation of esophageal cancer still predominates.

MATERIALS AND METHODS

Study population

HPV identification was carried out on 147 esophageal tissue samples taken from 91 patients treated between 2007 and 2010 in the Second Department of General and Gastrointestinal Surgery and Oncological Surgery of the Alimentary Tract, Medical University of Lublin, Poland. The study population consisted of 56 patients with ESCC (49 males aged 56.22 ± 7.34 years; 7 females aged 58.85 ± 7.77 years) and 35 controls with normal esophageal mucosa (30 males aged 33.73 ± 9.62 years; 5 females aged 45.91 ± 5.72 years). The detailed pathological characteristics of the ESCC patients are shown in Table 1. The controls were patients referred to our department due to other non-esophageal and non-oncological disorders with no gross and microscopic esophageal pathology as confirmed by endoscopy and histopathology. All investigated patients and controls were smokers.

The study was approved by the Ethics Committee of the Medical University of Lublin (0254/212/2007) and carried out in accordance with the principles of the Helsinki Declaration. Written informed consent was obtained from all the subjects.

Tissue samples in ESCC patients were taken during surgery from the specimen or during preoperative esophagoscopy. The first set of samples was taken from normal esophageal mucosa 10 cm proximal to the edge of the tumor (56 mucosa samples). A second set of samples was then taken from the tumor (56 tumor samples). Tissue samples in the controls were taken during esophagogastro-duodenoscopy from normal mucosa of the middle esophagus (35 control samples). Tissue samples were divided into two parts: one part was sent

Table 1 Clinico-pathological characteristics of esophageal squamous cell carcinoma patients investigated from Lublin region

Characteristic	*n* (%)
Gender	
Male	49 (87.5)
Female	7 (12.5)
Age (yr)	
> 50	46 (82.1)
< 50	10 (17.9)
Macroscopic type	
Ulcerative	10 (17.9)
Infiltrative	6 (10.7)
Ulcerative/infiltrative	21 (37.5)
Protruding	19 (33.9)
Grading	
G1	4 (7.1)
G2	43 (76.8)
G3	9 (16.1)
Staging	
Ⅱ	30 (53.6)
Ⅲ	19 (33.9)
Ⅳ	7 (12.5)
Tumor	
T2	19 (33.9)
T3	29 (51.8)
T4	8 (14.3)
Nodes	
N0	25 (44.6)
N1	31 (55.3)

for histopathological assessment and the other was stored in a freezer at -70 ℃ for molecular testing. Before molecular testing for HPV, histopathological assessment of all samples was carried out by two independent experienced pathologists. ESCC was confirmed in all tumor samples and no microscopic pathology was identified in the mucosa and control samples.

DNA isolation

Genomic DNA was isolated from esophageal cancer tissue and healthy esophageal mucosa by proteinase digestion and phenol extraction or using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions.

The esophageal tissue samples were homogenized following the addition of 1 mL buffer with the following composition: 0.01 mol Tris-HCl pH 7.5; 0.01 mol ethyl enediaminetetraacetic acid; 0.6% odium dodecyl sulfate. The homogenate was incubated for 30 min at room temperature. Then, K proteinase was added reaching a final concentration of 50 mg/L and incubated for 24 h at 37 ℃. Following incubation, half the volume of phenol: chloroform: isoamyl alcohol (in a ratio of 25:24:1) mixture was added to the solution; it was shaken for 15 min at room temperature and centrifuged for 15 min at 3000 *r*/min. The remaining volume of phenol, chloroform, isoamyl alcohol mixture was added to the water phase obtained, shaken energetically and then centrifuged. The above procedures were repeated until complete purification of DNA manifested as a lack of interphase was achieved. Then, half the volume of isopropylene alcohol and 0.1 volume of 3 mol acetate at pH 7.0 were added to the water phase obtained.

The DNA samples obtained in this manner were then rinsed in 80% ethanol and dissolved in distilled water after drying. The samples with dissolved DNA were stored at 20 ℃. Quantitative determination of the DNA obtained was carried out by spectrophotometry using an automatic spectrophotometer manufactured by Pharmacia Co. In order to determine the amount of DNA in a given sample, 1 μL of the sample was dissolved in 69 μL of re-distilled water and after calibration of the spectrophotometer, the sample was placed in its measuring chamber. After automatic processing of the data measured, the result was read in mg/L.

Table 2 Human papilloma virus identifying primers for polymerase chain reaction study

Primers	Region of amplification	Sequence 5′-3′	Product size (bp)
MY09	L1	CGTCCMARRGGAWACTGATC	450
MY11		GCMCAAGGWCATAAYAATGG M = A + C, R = A + G, W = A + T, Y = C + T	
HPV16/L1A	L1	GCCTGTGTAGGTGTTGAGGT	264
HPV16/L1B		TGGATTTACTCCAACATTGG	
ME12	HPV18/E6	CACGGCGACCCTACAAGC-TACCTG	404
ME50		TGCAGCACGAATTGGCACTG-GCCTC	

HPV: Human papilloma virus.

HPV identification

Genomic DNA was isolated from study tissues using the QIAamp DNA Midi Kit (QIAGEN) according to the manufacturer's protocol. HPV infection was identified following polymerase chain reaction (PCR) amplification of the *HPV* gene sequence, using primers MY09 and MY11 (Table 2) complementary to the genome sequence of at least 33 types of HPV viruses as described previously[13]. The reaction mixture contained 15 mg/L DNA and the following reagents: 0.5 U Taq DNA polymerase (Fermentas), PCR buffer and magnesium chloride at a final concentration of 1.5 mmol (Fermentas), primers at a final concentration of 0.25 mmol each and dNTPs (Promega) at a final concentration of 0.2 mmol. PCR products were run in 1.2% agarose gel electrophoresis with ethidium bromide and visualized in ultraviolet light. The product length was assessed according to the MassRuler marker (Fermentas). PCR products were randomly eluted from agarose gel using QIAGEN and sequenced in order to confirm the presence of the expected product. The sequencing results were computationally analyzed using the basic local alignment search tool database. These were sequenced in the Sequencing Laboratory Adam Mickiewicz University Poznań.

Statistical analysis

From the statistical analysis of the results obtained from

Table 3 Human papilloma virus status in esophageal squamous cell carcinoma patients investigated from Lublin region

HPV risk	*n* (%)	χ^2
Tumor samples (*n* = 56)		
High risk	5 (8.9)	
Low risk	19 (33.9)[b]	24.487
Others	4 (7.1)	
Total	28 (50.0)[d]	21.185
Mucosa samples (*n* = 56)		
High risk	3 (5.3)	
Low risk	12 (21.4)[b]	23.451
Others	6 (10.7)	
Total	21 (37.5)[d]	27.521
Control samples (*n* = 35)		
High risk	0 (0)	
Low risk	2 (5.7)	
Others	2 (5.7)	
Total	4 (11.4)	

[b]$P < 0.01$ *vs* human papilloma virus (HPV) high risk, Fisher test; [d]$P < 0.01$ *vs* control, Fisher test.

HPV (+) and HPV (-) patients, correlation tables containing structure indices were compiled. A method of statistical interference, verification of hypotheses based on homogeneity and independence test χ^2, was used. The differences between groups of ESCC samples according to analyzed clinico-pathological parameters were compared using the Mann-Whitney *U* test or the Kruskal-Wallis test depending on the distribution of the grouping variable. Statistical significance was found at $P < 0.05$. The statistical analyses were performed on an IBM PC, using SPSS 8.0 PL for Windows 95 and Statistica 5.0.

RESULTS

The results of molecular testing which evaluated the occurrence of HPV in 147 esophageal tissue samples from patients with ESCC and controls are shown in Table 3. In tumor samples, HPV DNA was identified in 28 of 56 samples (50%). High risk HPV DNA (16 or/and 18) was found in 5 of 56 samples (8.9%), low risk in 19 of 56 samples (33.9%) and other types of HPV (37, 81, 97, CP6108) in 4 of 56 samples (7.1%). In mucosa samples, HPV DNA was isolated in 21 of 56 samples (37.5%). High risk HPV DNA was confirmed in 3 of 56 samples (5.3%), low risk HPV DNA in 12 of 56 samples (21.4%), and other types of HPV in 6 of 56 samples (10.7%). In control samples, HPV DNA was identified in 4 of 35 samples (11.4%). In control samples, no high risk HPV DNA was isolated. Two samples contained low risk HPV DNA (5.7%) and other types of HPV were found in 2 of 56 cases (5.7%).

The occurrence of HPV DNA in ESCC tissue and unaffected esophageal mucosa taken from patients with esophageal cancer was significantly higher than in control samples taken from healthy esophageal mucosa [28 of 56 (50%) *vs* 4 of 35 (11.4%), $P < 0.001$]. In esophageal cancer patients, both in tumor and mucosa samples, the predominant HPV phenotype was low risk HPV DNA, and was isolated approximately 4 times more frequently than high risk phenotypes. This difference reached statistical significance [19 of 56 (33.9%) *vs* 5 of 56 (8.9%), $P < 0.001$].

Table 4 Clinico-pathological characteristics of esophageal squamous cell carcinoma patients investigated from Lublin region

Characteristic	HPV positive		HPV high risk		HPV low risk	
	n (%)	*P* value	*n* (%)	*P* value	*n* (%)	*P* value
Gender						
Male	23 (46.9)	0.4435[1]	2 (4.1)	0.0294[1]	17 (34.7)	0.1254[1]
Female	5 (71.4)		3 (42.9)		2 (28.6)	
Age (yr)						
> 50	25 (54.4)	0.7274[1]	3 (6.5)	0.2332[1]	18 (39.1)	0.2217[1]
< 50	3 (30.0)		2 (20.0)		1 (10.0)	
Macroscopic type						
ULC	2 (20.0)	0.0293[2]	0 (0.0)	0.1125[2]	2 (20.0)	0.1448[2]
INF	5 (83.3)		1 (16.7)		4 (66.7)	
ULC/INF	15 (71.4)		3 (14.3)		9 (42.9)	
PROT	6 (31.6)		1 (5.3)		4 (21.1)	
Grading						
G1	3 (75.0)	0.3234[2]	1 (25.0)	0.2638[2]	2 (50.0)	0.2911[2]
G2	19 (44.2)		3 (7.0)		15 (34.9)	
G3	6 (66.7)		1 (11.1)		2 (22.2)	
Staging						
Ⅱ	11 (36.7)	0.2720[2]	3 (10.0)	0.6106[2]	6 (20.0)	0.3537[2]
Ⅲ	14 (73.7)		2 (10.5)		10 (52.6)	
Ⅳ	3 (42.9)		0 (0.0)		3 (42.9)	
Tumor						
T2	11 (57.9)	0.3458[2]	3 (15.8)	0.1013[2]	7 (36.8)	0.3125[2]
T3	15 (51.7)		1 (3.5)		11 (37.9)	
T4	2 (25.0)		1 (12.5)		1 (12.5)	
Nodes						
N0	12 (48.0)	0.7604[1]	3 (12.0)	0.2484[1]	7 (28.0)	0.4626[1]
N1	16 (51.6)		2 (6.5)		12 (38.7)	

[1]Mann-Whithey *U* test; [2]Kruskal-Wallis test. HPV: Human papilloma virus; ULC: Ulcerative; INF: Infiltrative; PROT: Protruding.

A higher prevalence of HPV DNA was identified in female patients (71.4% *vs* 46.9%). Accordingly, high risk HPV phenotypes were isolated more frequently in female patients and this difference reached statistical significance [3 of 7 (42.9%) *vs* 2 of 49 (4.1%), $P < 0.05$] (Table 4). Of the pathological characteristics, only an infiltrative pattern of macroscopic tumor type significantly correlated with the presence of HPV DNA in ESCC samples [20 of 27 (74.1%) *vs* 8 of 29 (27.6%), $P < 0.05$] (Table 4). The occurrence of total HPV DNA and both HPV high or low risk phenotypes did not significantly differ with regard to particular grades of cellular differentiation, phases in depth of tumor infiltration, grades of nodal involvement and stages of tumor progression. The detailed distribution of total HPV DNA, high and low risk HPV phenotypes according to particular clinico-pathological characteristics are shown in Table 4.

DISCUSSION

The role of HPV in the etiology of ESCC is not clear. To date, over 70 studies have tested for HPV in esophageal cancer tissue[13] and the reported HPV detection rate ranged from 0% to 67%[8-12]. The data from China[14] pro-

vided the most convincing evidence to date that HPV is not directly involved in ESCC carcinogenesis, at least in China. HPV DNA contamination cannot be ruled out as an explanation for high HPV occurrence in ESCC tissue studies with less stringent tissue procurement and processing protocols. A few possible reasons for this variation between studies include small study sites, different HPV detection assays, interlaboratory variability and suboptimal sample collection and handling leading to contamination[1,15].

The incidence of HPV infection in ESCC patients was significantly higher in comparison to controls with normal esophageal mucosa. In our study we identified HPV DNA in 50% of ESCC patients, but contrary to the Chinese data[1], the detected HPV types had low oncogenic potential. A high risk 16 or/and 18 HPV DNA type was only detected in 5 of 56 cases (8.92%). HPV types with low oncogenic potential were found 4 times more frequently than high risk HPV types both in tumor tissue and distant mucosa in patients with ESCC. These differences reached statistical significance.

HPV type 16 was detected in 16% of ESCC patients in Uppsala, Sweden[16] and this was higher than in our population. Xu *et al*[17] reported that the detection rate of infection with all HPV types (16, 18 and 58) in ESCC tissue in Chinese patients was 50%. This number corresponded well with data observed in our study. When investigated by nested polymerase chain reaction, HPV DNA was identified in all ESCC tumor tissue in German patients[18]. Concomitantly, another potentially oncogenic virus considered to be involved in ESCC malignant transformation was EBV DNA which was detected in 35% of these patients.

A search of the most frequently used databases (Medline, PubMed) using the terms esophageal squamous cell carcinoma, HPV and Poland, revealed a lack of studies investigating the occurrence of HPV DNA in ESCC in the Polish population. To our knowledge this is the first report from Poland elaborating this issue.

Analysis of the prevalence of HPV DNA in ESCC samples according to different clinico-pathological parameters revealed that HPV was more frequently isolated in female patients and in tumors showing an infiltrative growth pattern (Table 4). In the majority of studies, investigators did not confirm statistically significant differences between HPV-positive and HPV-negative cases with regard to clinical and pathologic findings[19-26]. Nevertheless, Antonsson *et al*[20] reported that patients with HPV-positive ESCC tumors were younger than those with HPV-negative tumors and had higher body mass index. In turn, Harrera *et al*[21] in a Mexican population found that male patients were most commonly affected and the male:female ratio in HPV-positive ESCC increased two-fold in comparison with HPV-negative cases. Dreilich *et al*[16] found a significantly higher rate of the HPV 16 phenotype in advanced compared with localized cancer but this did not translate into prognostic significance. Although isolated data may suggest some association between HPV and aggressiveness and prognosis of ESSC, generalized and coherent conclusions concerning the influence of HPV and its particular phenotypes on clinico-pathological characteristics of ESCC cannot be drawn.

In summary, we identified low risk HPV DNA types in ESCC patients significantly more frequently than other types (37, 81, 97, CP6108). This may suggest that low risk HPV types could be one of the co-activators or/and co-carcinogens in complex, progressive, multifactorial and multistep esophageal carcinogenesis. HPV DNA occurrence does not preclude development of the squamous cell phenotype of esophageal carcinoma.

COMMENTS

Background

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related death worldwide. Overall survival rates range between 5% and 16%. Although there is a marked tendency for increasing incidence rates of esophageal adenocarcinoma in industrialized Western countries, the world leading pathological type of esophageal cancer is still squamous cell carcinoma. Marked geographic, cultural and ethnic variations suggest the importance of environmental influences in the development of this condition. In Europe, the incidence of esophageal cancer is increasing and seems to parallel the decrease in gastric cancer incidence. Poland is a low-incidence country with the reported age-standardized annual incidence of 4.5 and 0.7/100.0, for men and women, respectively.

Research frontiers

Esophageal carcinoma is multifactorial in origin and typically arises after many years of tobacco and alcohol abuse. Other causes include chronic mucosal injury due to hot beverages and malnutrition. An etiologic role of different microorganisms such as fungi, bacteria and viruses has also been proposed for the pathogenesis of esophageal squamous cell cancer (ESCC). Of these microorganisms, human papilloma virus (HPV), cytomegalovirus and Epstein-Barr virus have been investigated. The role of HPV in the etiology of ESCC has been debated for the past 30 years.

Innovations and breakthroughs

In this study, the authors found HPV DNA in 50% of ESCCs. High risk HPV phenotypes (16 or/and 18) were found in 8.9%, low risk in 33.9% and other types of HPV (37, 81, 97, CP6108) in 7.1% of the patients. Low risk HPV DNA types were identified significantly more frequently than other types (37, 81, 97, CP6108). This may suggest that low risk HPV types could be one of the co-activators or/and co-carcinogens in complex, progressive, multifactorial and multistep esophageal carcinogenesis.

Applications

The data on the prevalence of different types of HPV may contribute to the design of future strategies for the prevention of HPV-related malignancies, including the development of effective vaccines.

Terminology

HPVs are DNA viruses that infect basal skin and mucosal cells. HPVs are categorized according to their cervical oncogenicity-based risk, with high, probably high, low and undetermined risks.

Peer review

In this manuscript, the authors investigated the prevalence of HPV in esophageal squamous cell carcinoma in Poland. Overall positive rate of HPV was similar to the previous reports. However HPV DNA type was different from other countries. Since this is the first report to investigate about relationship between HPV DNA type and esophageal squamous cell carcinoma, this study is certainly valuable in this research field.

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BRIEF ARTICLE

Intussusception due to inflammatory fibroid polyp: A case report and comprehensive literature review

Sami Akbulut

Sami Akbulut, Department of Surgery, Diyarbakir Education and Research Hospital, 21400 Diyarbakir, Turkey
Author contributions: Akbulut S performed the surgical procedure and review of the literature, as well as undertaking a comprehensive literature search.
Correspondence to: Dr. Sami Akbulut, MD, Department of Surgery, Diyarbakir Education and Research Hospital, 21400 Diyarbakir, Turkey. akbulutsami@gmail.com
Telephone: +90-412-2580052 Fax: +90-412-2580050
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Abstract

AIM: To give an overview of the literature on intussusception due to inflammatory fibroid polyp (IFP).

METHODS: We present a new case of ileal intussusception due to IFP and a literature review of studies published in English language on intussusception due to IFP, accessed *via* PubMed and Google Scholar databases. For the search, the keywords used were: intussusception, IFP, intussusception and IFP, intussusception due to IFP, and IFP presenting as intussusception. The search covered all articles from 1976 to November 2011.

RESULTS: We present a 38-year-old woman who was admitted 10 d after experiencing abdominal pain, vomiting, and nausea. Ultrasonography demonstrated small bowel intussusception. An ileal intussusception due to a mass lesion 50 cm proximal to the ileocecal junction was found during laparotomy. Partial ileal resection and anastomosis were performed. A diagnosis of ileal IFP was made based on the immunohistochemical findings. In addition, a total of 56 reports concerning 85 cases of intussusception due to IFP meeting the aforementioned criteria was included in the literature review. The patients were aged 4 to 81 years (mean, 49 ± 16.2 years); 44 were women (mean, 51.8 ± 14.3 years) and 41 were men (mean, 46 ± 17.5 years). According to the location of the IFP, ileal intussusception was found in 63 patients, while 17 had jejunal, three had colonic, and two had ileojejunal intussusception.

CONCLUSION: Although IFPs are rare and benign, surgery is the only solution in case of intestinal obstruction. Differential diagnosis should be made *via* immunohistochemical examination.

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Key words: Intussusception; Inflammatory fibroid polyp; Vanek's tumor; Immunohistochemical stain

Peer reviewer: Dr. Mehmet Yilmaz, Division of Liver Transplantation, Department of Surgery, Inonu University Faculty of Medicine, 44280 Malatya, Turkey

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INTRODUCTION

Intussusception occurs when a more proximal portion of the bowel (intussusceptum) invaginates into the more distal bowel (intussuscipiens)[1-5]. The pathomechanism is thought to involve altered bowel peristalsis at the intraluminal lesion, which is then a lead point for the intussusceptum[1]. Although intussusception is a common condition in children, it is rare in adults. Adult intussusception differs from pediatric intussusception in various respects, including etiology and clinical characteristics[1-4]. Adult intussusception represents 5%-16% of all cases of intussusception and 1%-5% of all cases of intestinal

obstruction in adults[1-3,5-11]. In children, 90% of cases are idiopathic, whereas 70%-90% of cases of adult intussusception are secondary to an underlying pathology, with approximately 65% being due to benign or malignant neoplasms[1,4,12]. Non-neoplastic processes represent 15%-25% of cases, while idiopathic intussusception accounts for about 10% of cases[2,5,6,12-14]. The presentation of pediatric intussusception is often acute with sudden onset of intermittent colicky pain, vomiting, and bloody mucoid stools, and the presence of a palpable mass. In contrast, adults may present with acute, subacute, or chronic nonspecific symptoms[2-4].

Up to 90% of occurrences in adults have a lead point, defined as a well-definable pathological abnormality. Most lead points in the gastrointestinal tract involve primary or metastatic malignancy, lipomas, leiomyomas, adenomas, neurofibromas, postoperative adhesions, Meckel's diverticulum, foreign bodies, vascular anomalies, lymphoid hyperplasia, trauma, celiac disease, cytomegalovirus colitis, lymphoid hyperplasia secondary to lupus, Henoch-Schönlein purpura, Wiskott-Aldrich syndrome, appendiceal stump, or inflammatory fibroid polyps (IFP)[1,4,14-16].

IFPs are rare, benign, tumor-like lesions of the gastrointestinal tract. They are most commonly localized to the gastric antrum, but can develop anywhere in the gastrointestinal tract. The small bowel is the second most common site of origin, where IFPs usually present as intussusception or obstruction[15,17-20]. Here, we present a rare case of ileoileal intussusception due to IFP in a 38-year-old woman. A review of 85 cases reported in the English literature is also presented.

MATERIALS AND METHODS

Here, we present and discuss a new case of ileo-ileal intussusception caused by IFP. In addition, a search of the English-language medical literature using PubMed and Google Scholar was conducted for articles related to gastrointestinal intussusception due to IFP; the key words used were intussusception, IFP, inflammatory fibrous tumor, intussusception and IFP, intussusception due to IFP, and IFP presenting as intussusception. The search covered all articles from 1976 to November 2011. If there were any missing data, the corresponding authors of the articles in question were contacted by email. Articles containing adequate information, such as publication year, patient age, sex, duration of complaint, radiological tools, presence of palpable mass, surgical approach, tumor location, and tumor size, were included, while studies and comment articles with insufficient clinical and demographic data were excluded.

RESULTS

Case report

A 38-year-old female patient presented to our emergency clinic with a 10-d history of abdominal pain, vomiting, and nausea. Upon auscultation of the abdomen, bowel sounds were found to be consistent with mechanical bowel obstruction. Physical examination revealed muscular defense and rebound tenderness. Digital rectal examination did not reveal the presence of feces, mucus, or blood. Laboratory investigations showed blood urea nitrogen, 38 mg/dL (normal: 5-23 mg/dL); creatinine, 1.7 mg/dL (normal: 0.6-1.2 mg/dL); and C-reactive protein, 58 mg/L (normal: 0-0.5 mg/L). Blood cell counts revealed a leukocyte count of 8.77/L (normal: 4-11.000/L), a hemoglobin level of 11.4 g/dL (normal: 12-16 g/dL), and a platelet count of 275/L (150-450 000/L). Other serum parameters were within the normal limits. An upright plain abdominal film revealed small bowel obstruction, with marked small bowel air-fluid levels (Figure 1). Abdominal ultrasound revealed a hypoechoic, round, soft tissue mass measuring 35 mm × 38 mm at the end of a dilated, thick-walled ileal loop at the right iliac fossa. The clinical symptoms and ultrasonographic images were consistent with intussusception or mechanical intestinal obstruction caused by a tumoral mass. Thus, laparotomy with midline incision was performed. On exploration, an ileoileal intussusception was found 50 cm proximal to the ileocecal valve. The intussuscepted intestinal segments were obstructing the lumen, causing dilatation in the intestine proximal to the intussusception (Figure 2). Segmental resection of the intussuscepted ileum and end-to-end anastomosis were performed. Macroscopically, the resected segment of the ileum was 20 cm in length. Histopathological examination showed an IFP measuring 4 cm × 4 cm, composed of an edematous stroma containing spindle-shaped stromal cells, lymphoid nodules and eosinophils. On immunohistochemical analysis, the



Figure 1 Upright plain abdominal radiography revealed small bowel obstruction with marked small bowel air-fluid levels.



Figure 2 Intraoperative photograph of ileoileal intussusception.

spindle cells were negative for CD117, smooth muscle actin, desmin, S100 protein and CD34. A diagnosis of IFP (Vanek's polyp) of the ileum was made. The patient recovered without complications and has remained well and symptom-free until at least 6 mo after discharge.

Literature review

The English-language medical literature published between 1976 and November 2011 was searched using PubMed and Google Scholar. A total of 56 reports concerning 85 cases of intussusception due to IFP meeting the aforementioned criteria were included in this review. The patients were aged from 4 to 81 years (mean, 49 ± 16.2 years); 44 were women (mean, 51.8 ± 14.3 years) and 41 were men (mean, 46 ± 17.5 years). According to the localization of the IFP, 63 patients had ileal intussusception, while 17 had jejunal, three had colonic, and two had ileojejunal intussusception. The clinicopathological characteristics of the 85 patients are summarized in Table 1.

DISCUSSION

Intussusception was first described by Barbette in 1674 and later by Hunter in 1789. Sir Jonathan Hutchinson was the first surgeon who operated on intussusception in a child in 1871[2,5,6,17,21]. Intussusception, defined as invagination of a proximal part of small bowel along with its mesentery into an adjacent segment, can lead to impaired peristalsis, obstruction, and possible vascular compromise[3,6,11,15,17]. The precise mechanism of intestinal intussusception remains unclear. However, it is believed that any lesion in the bowel wall or irritant within the lumen that alters normal peristaltic activity, forming leading edges for the intussusceptum, may initiate invagination. Ingested food and subsequent peristaltic activity of the bowel produces an area of constriction above the stimulus and relaxation below, thus telescoping the lead point through the distal bowel lumen[3-5].

Intussusceptions have been classified according to location into three major categories, i.e., enteroenteric, ileocolic or ileocecal, and colocolic. Enteroenteric intussusception is confined to the small bowel. In ileocolic intussusception, the ileum invaginates through the ileocecal valve. Colocolic intussusception is confined to the colon[1,5]. Adult intussusception occurs more frequently in the small bowel (50%-88%) than in the large bowel (12%-50%)[1].

Intussusception can be classified according to the underlying etiological factors as primary (idiopathic) or secondary (benign or malignant lesion). Primary or idiopathic adult intussusception accounts for about 10% of cases and is more likely to occur in the small intestine. Secondary intussusception, which is more common in the adult population, is associated with a pathological condition involving a lead point. The etiologies of intussusception in the small bowel and the colon are quite different. In the small intestine there is a predominance of benign processes, with up to 90% of cases, including hamartomas, lipomas, leiomyoma neurofibromas, adenomas, Peutz-Jeghers syndrome, IFP, adhesions, Meckel's diverticulum, lymphoid hyperplasia, trauma, celiac disease, intestinal duplication, Henoch-Schönlein purpura, appendiceal stump, and tuberculosis. Malignant lesions (either primary or metastatic) account for 14%-47% of cases of intussusception in the small intestine. On the other hand, intussusception occurring in the large bowel is more likely to have a malignant etiology and accounts for 43%-80% of cases[4,8,12,21]. Colon adenocarcinoma is the most important cause of malignant large bowel intussusception. Benign lesions provoking large bowel intussusception include lipomas, leiomyomas, adenomatous polyps and endometriosis. We reviewed the published literature regarding intussusception, and the underlying cause in most cases of enteroenteric intussusception was benign, whereas it was mostly malignant in the ileocolic and colocolic cases. That is, the potential for malignancy increased from proximal to distal intussusception. Thus, the most important factors in the surgical decision process in an adult patient without a histopathological diagnosis in the preoperative period and intussusception was detected during a surgical operation are the location and size of the mass and viability of the invaginated segment. Benign tumors, often lipomas, are the most common specific causes of adult intussusception. IFPs have been reported as a rare cause of adult intussusception[22,23]. As the main topic of this report is intussusception due to IFPs, a discussion of the general features of gastrointestinal IFPs is presented below.

IFPs are rare, idiopathic pseudotumorous lesions of the gastrointestinal tract, which were first described by Vanek in 1949 as an eosinophilic submucosal granuloma. In that first report of six gastric lesions, Vanek called attention to the inflammatory nature of the lesions and their submucosal origin[24-28]. Various names for IFPs have been suggested, including eosinophilic granuloma, submucosal fibroma, hemangiopericytoma, inflammatory pseudotumor and fibroma. However, the term IFP first proposed by Helwig and Ranier in 1953 for gastric polyps has gained acceptance for similar lesions throughout the gastrointestinal tract[15,16,26,29,30].

IFPs can develop in many different locations in the gastrointestinal tract. The most common site is the gastric antrum (66%-75%), followed by the small bowel (18%-20%), colorectal region (4%-7%), gallbladder (1%), esophagus (1%), duodenum (1%), and appendix (< 1%)[18,21,26,31,32]. However, the ileal segment is the most common site where these polyps cause intussusception[21].

The precise etiopathogenesis of IFPs remains unknown, but trauma, allergic reaction, genetic tendency, bacterial, physical, chemical and even metabolic stimuli have been suggested as initiators of the process. The more common occurrence of these lesions in the stomach, with its coarse food content and active muscular contractions, supports a traumatic etiology, but it is dif-

Table 1 General characteristics of 85 cases of intussusception due to inflammatory fibroid polyp reported between 1976 and 2011

Ref.	Age	Sex	Duration of complaint	Palpable mass	Radiologic tools	Surgical approach	Tumor location	Tumor size (cm)
Yakan *et al*[4]	60	F	NS	NS	Urgent	S.Resection	Ileum	NS
	56	F	NS	NS	CT	R.Hemicol	Ileum	NS
	28	F	NS	NS	Urgent	S.Resection	Jejenum	NS
	62	F	NS	NS	CT	S.Resection	Ileum	NS
Mohamud *et al*[8]	70	M	6 yr	(-)	CT	R.Hemicol, Lap	Ileum	3
O'Kane *et al*[9]	65	F	1 mo	(-)	CT	S.Resection	Ileum	3.5
Coulier *et al*[10]	58	M	Several weeks	(+)	CT	Ileocecal resec.	Ileum	6
Malik *et al*[11]	31	M	3 d	(+)	US	R.Hemicol	Ileum	3.5
Karamercan *et al*[12]	56	M	1 d	(+)	US, CT	S.Resection	Ileum	4
Cawich *et al*[13]	50	M	3 d	(-)	Urgent	S.Resection	Jejenum	3
Hsieh *et al*[14]	56	M	NS	(-)	CT	S.Resection	Ileum	2.5
Akbulut *et al*[15]	73	F	5 d	(-)	US	S.Resection	Ileum	11
Gara *et al*[16]	76	F	NS	(-)	CT	S.Resection	Ileum	5.5
Toydemir *et al*[17]	54	M	2 mo	(-)	CT	W.Resection	Ileum	4
Szczepanow *et al*[18]	72	F	2 d	(-)	US	S.Resection	Ileo-jejunal	3
Ijaz *et al*[19]	10	M	3 d	(+)	US, Barium	S.Resection	Colon	NS
Bradley *et al*[20]	62	M	4 mo	(-)	Enteroclys	S.Resection	Ileum	8
Nonose *et al*[21]	56	F	45 d	(-)	CT	S.Resection	Ileum	4.5
Singhal *et al*[22]	65	M	NS	(-)	NS	S.Resection	Ileum	3
	45	F	NS	(-)	NS	S.Resection	Jejenum	1.5
Balci *et al*[23]	71	F	NS	(+)	MRI	S.Resection	Ileum	1.5
Morales-Fu *et al*[24]	42	M	8 d	(-)	CT	S.Resection	Ileum	3
Ruffolo *et al*[25]	44	F	3 d	NS	CT	S.Resection, Lap	Ileum	3.7
Costamagna *et al*[26]	62	M	3 wk	NS	NS	S.Resection	Ileum	4
Deschamps *et al*[27]	22	M	NS	(-)	CT	S.Resection	Ileum	3
Shih *et al*[28]	66	M	4 d	(-)	Urgent	S.Resection	Jejenum	5
Korkmaz *et al*[29]	30	M	NS	(+)	US, CT	S.Resection	Ileum	3
	60	F	NS	(-)	US, CT	S.Resection	Ileum	4
El Hajj *et al*[30]	52	F	10 d	(-)	CT	S.Resection, Lap	Jejenum	3.5
Dicle *et al*[31]	22	M	2 mo	(-)	US, Barium, CT	S.Resection	Ileum	NS
Bandyopad *et al*[32]	64	F	3 wk	(-)	US, Barium	S.Resection	Ileum	4
de la Plaza *et al*[33]	63	M	2 mo	(+)	US, Barium	R.Hemicol	Colon	3.5
Kuesterman *et al*[34]	34	F	5 wk	NS	CT	S.Resection	Jejenum	5
Farrell *et al*[35]	70	M	12 h	(-)	Urgent	R.Hemicol.	Ileum	2
Ling *et al*[36]	56	F	8 wk	NA	NA	NA	Jejenum	NA
Kim *et al*[37]	52	F	10 d	NA	NA	NA	Jejenum	NA
Aanestad *et al*[38]	31	M	36 h	(-)	Urgent	S.Resection	Ileum	NS
Nkanza *et al*[39]	NS	F	NS	NS	NS	S.Resection	Ileum	3.5
	40	F	NS	NS	NS	S.Resection	Ileum	5
	45	M	NS	NS	NS	S.Resection	Ileum	8
	50	M	NS	NS	NS	S.Resection	Ileum	4
	40	F	NS	NS	NS	S.Resection	Ileum	2.5
	45	F	NS	NS	NS	S.Resection	Ileum	8
	35	F	NS	NS	NS	S.Resection	Ileum	4
	62	M	NS	NS	NS	S.Resection	Ileum	4
	NS	F	NS	NS	NS	S.Resection	Ileum	3
	30	F	NS	NS	NS	S.Resection	Ileum	3
	55	F	NS	NS	NS	S.Resection	Ileum	4
	4	M	NS	NS	NS	S.Resection	Ileum	NS
Bays *et al*[40]	54	F	8 mo	(-)	US, CT, Ecl	S.Resection	Ileum	3.5
	17	M	2 mo	(-)	US, CT, Ecl	S.Resection	Jejenum	3
Santos Gda *et al*[41]	37	F	NS	NS	NS	S.Resection	Ileum	4
	29	M	NS	NS	NS	S.Resection	Ileum	2
	58	M	NS	NS	NS	S.Resection		2.5
Dalton *et al*[42]	17	M	5 mo	(-)	Barium	S.Resection	Jejenum	2.8
Gonul *et al*[43]	48	M	1 d	(-)	Urgent	S.Resection	Ileum	5
Miyata *et al*[44]	64	F	6 wk	(+)	US, CT, Enteros	S.Resection	Jejenum	4.5
Dawson *et al*[45]	47	F	12 h	(-)	Urgent	S.Resection	Ileum	3.5
Sampson *et al*[46]	43	F	4 d	(+)	US	S.Resection	Ileum	3
	60	F	5 d	(-)	Urgent	S.Resection	Ileum	3.5
Carlén *et al*[47]	55	F	3 d	(-)	Urgent	S.Resection	Ileum	2
Martin-Lor *et al*[48]	28	F	NS	NS	Urgent	R.Hemicol	Ileum	NS
Savargaon *et al*[49]	52	F	7 mo	(-)	CT	S.Resection	Ileum	3
	42	F	4 d	(-)	CT	Ileocecal resec.	Ileum	2.5
Parasi *et al*[50]	35	F	NA	NA	NA	S.Resection	Ileum	4

Jabar *et al*[51]	34	M	2 d	(+)	US	S.Resection	Ileo-jejunal	3
	47	M	4 mo	(-)		R.Hemicol	Ileum	NS
Vijayaragh *et al*[52]	20	F	3 wk	(-)	DL	S.Resection	Jejenum	6
Topaloglu *et al*[53]	56	M	10 d	(-)	US, CT	S.Resection	Jejenum	5
Sah *et al*[54]	45	M	2 wk	(-)	NA	NA	Jejenum	NA
Zager *et al*[55]	54	M	NS	(-)		Ileocecal resec., Lap	Ileum	2.5
	46	F	3 mo	(-)	CT	S.Resection	Jejenum	3
	36	M	2 d	(-)	CT	S.Resection, Lap	Ileum	3.5
Winkler *et al*[56]	61	M	7 d	(-)	Urgent	S.Resection	Jejenum	4
Rubinstein *et al*[57]	31	M	1 wk	(-)	Barium	S.Resection	ileum	3
	28	M	2 d	(-)	Barium	S.Resection	Ileum	6.5
Benjamin *et al*[58]	62	M	1 wk	(+)	Barium	R.Hemicol	Ileum	7
	56	M	6 wk	(-)	Barium	R.Hemicol	Ileum	7
	64	M	8 wk	(-)	Barium	S.Resection	Ileum	7
	61	M	NS	(-)	Barium	R.Hemicol	Ileum	5.5
McGregor *et al*[59]	38	F	12 h	(+)	NP	S.Resection	Ileum	2.8
Muniz *et al*[60]	56	F	5 mo	(-)	Scintigraphy	S.Resection	Jejenum	6
Widgren *et al*[61]	70	F	NS	NS	NS	S.Resection	Ileum	3
	77	F	NS	NS	NS	S.Resection	Ileum	10
Pritchett *et al*[62]	81	F	1 mo	(+)	Urgent	NS	Ileum	4

F: Female; M: Male; NP: Not-performed; S.Resection: Segmental resection; Col: Colonoscopy; NS: Not-stated; NA: Not-available; R.Hemicol: Right hemicolectomy; Ecl: Enteroclysis; DL: Diagnostic laparoscopy; US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging; Ileocecal resec.: Ileocecal resection; Lap: Laparoscopy; W.Resection: Wedge resection.

ficult to account for lesions in the lower ileum on this basis[33-39].

IFPs are usually asymptomatic, and are typically identified during endoscopic procedures and laparotomy. When they are symptomatic, the clinical symptoms depend on the location and size of the tumor[9,27,40-42]. Abdominal pain is the main symptom in patients with lesions in the stomach. Intussusception and obstruction are the most frequent initial symptoms when the polyp is located in the small intestine[10,26-28,40,43,44]. Other gastrointestinal symptoms, such as vomiting, diarrhea, bloody stools, tenesmus, and alterations in bowel habits, are also seen although their frequencies are low[30]. At the time of diagnosis, most IFPs usually measure between 2 and 5 cm in diameter. However, giant IFPs up to 20 cm in diameter have also been reported[9,10,15-17,26,27,33]. IFPs usually present in the sixth or seventh decade, but cases have been reported over a wide age range from 5 mo to 92 years old[35-38,45-47]. There is a slight predominance of the condition in men. Macroscopically, these tumors are pedunculated or sessile, measure 0.2-20 cm in diameter, arise from the submucosa, and project into the bowel lumen. The mucosal surface is usually ulcerated and pale. Microscopically, it is composed of mononuclear, spindle-shaped cells, forming a confused or whirl-like structure. The inflammatory infiltration also includes blood vessels, eosinophils, lymphocytes, macrophages, and mastocytes[10,14,18,19,25-27].

IFPs can mimic several other tumor and non-tumor processes of the gastrointestinal tract. Differential histopathological diagnoses include spindle cell lesions, such as inflammatory fibrosarcoma, spindle-cell carcinoids, and gastrointestinal stromal tumors (GISTs). Differentiation can be difficult, especially between IFPs and GISTs[10,15-17,22,26,48]. GISTs are common in the stomach and frequently present as polypoid masses. In the intestine, these tumors can present with intussusception similar to IFP. Immunohistochemistry is used to distinguish between IFPs and GISTs. Both tumors are positive for CD34 and vimentin, but GISTs are positive for CD117, while IFPs are not[13,24,26,49].

The preoperative diagnosis of intussusception is controversial. An accurate diagnosis is based on a good medical history, thorough physical examination, and specific imaging modalities, such as X-ray, ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), enteroclysis, endoscopic procedures, angiography, and capsule endoscopy[6,50-55]. Typically, abdominal X-ray examination is the first diagnostic tool used, because obstructive symptoms dominate the clinical picture in most cases[56-60]. Barium enema was the gold standard for diagnosis of intussusception until the mid-1980s when studies established that US could be used to diagnose the condition accurately. Around the same time, it was found that air could be used to diagnose and treat intussusception[5,61,62]. Today, enteroclysis is rarely used in the diagnosis of intussusception. This invasive double-contrast imaging method requires intubation using a special catheter with a double lumen and balloon to the proximal jejunum and is performed under fluoroscopy, MRI, or CT imaging. Although enteroclysis shows not only the inside of the lumen but also has high sensitivity and specificity for revealing small and mucosal lesions, its invasive nature limits its use[40]. Colonoscopy is useful only in cases in which colonic involvement is strongly suspected, and it allows the lesion to be diagnosed and biopsied. On colonoscopy, intussusception is seen as an intraluminal mass directed centrally and distally. However, diagnosis is rarely made by colonoscopy, and the diagnosis is usually made during surgery[4,48].

Capsule endoscopy and digital balloon endoscopy are newer means of diagnosing various gastrointestinal disorders[4,7]. Capsule endoscopy is a non invasive diagnostic test used to locate the source of gastrointestinal bleed-

ing and to identify the causes of other intestinal disorders, including intussusception and various tumors. On capsule endoscopy, intussusception has been reported to appear as mass lesion of the small bowel. Although obstructive symptoms are contraindicated for capsule endoscopy, this new method for evaluating the small bowel could be helpful in cases with long-standing abdominal pain and negative results on radiological examination, CT, or barium studies, to exclude the possibility of malignancy. Double-balloon enteroscopy, also known as "push-and-pull" enteroscopy, can be used to examine approximately 70-150 cm of the small bowel, and double balloon enteroscopy can examine the full length of the small bowel, both antegrade and retrograde[7,44]. Diagnostic laparoscopy (DL) may assist in the diagnosis of intussusception in cases in which the diagnosis is suspected but not confirmed by preoperative workup. Moreover, DL can help to establish the cause and is less traumatic than an exploratory laparotomy. US is considered a useful tool in the diagnosis of intussusception in both pediatric and adult cases[5-7,15,46]. Its classical imaging features include the target or doughnut sign in the transverse view and the pseudokidney, sandwich, or hayfork sign in longitudinal view[4,5,7,40]. However, obesity and the presence of a large amount of air in the distended bowel loops can limit image quality and subsequent diagnostic accuracy. Overall, US has a sensitivity of 98%-100% and a specificity of 88%-89% for diagnosing intussusception[3,17,29,40]. Abdominal CT is currently considered the most sensitive radiological method for confirming intussusception, with a reported diagnostic accuracy of 58%-100%[1-3,5,7,21]. On CT, a bowel-within-bowel configuration suggested by mesenteric fat and vessels compressed between the walls of the small bowel is pathognomonic of intussusception[3-6]. In contrast to US, CT is unaffected by the presence of gas in the bowel lumen. Therefore, we suggest that all patients presenting with an intestinal obstruction should undergo a CT scan as a routine diagnostic measure[3]. MRI is not applied routinely in diagnosis of intussusception in either children or adults. However, MRI can contribute to the radiological diagnosis of intussusception by demonstrating the "bowel-within-bowel" or "coiled-spring" appearance. This pattern can be seen more clearly with the use of the recently developed half-Fourier acquisition single-shot turbo spin-echo MRI modality. A polyp can be detected as a leading point using a combination of breathing-independent T2-weighted MRI and gadolinium-enhanced breath-hold T1-weighted imaging[4,23,30,34,40].

The appropriate management of adult intussusception remains controversial, with the debate focusing mostly on the issue of primary *en bloc* resection *vs* initial reduction followed by more limited resection. Reduction by surgery before resection may theoretically permit more limited resection; however, the risk of potential intraluminal seeding or venous tumor dissemination during the manipulation of a malignant lesion should also be taken into consideration. The incidence of malignancy as the cause of small intestinal intussusception ranges from 1% to 47%, and the majority of lesions are metastatic. Therefore, recent reports have recommended initial reduction of externally viable small bowel prior to resection. The likelihood of cancer in ileocolic and colocolic intussusception is 43%-100%. The vast majority of these lesions arise as a primary lesion, in which resection without reduction is recommended[1,2,4,5,48].

COMMENTS

Background

Intussusception occurs when a more proximal portion of the bowel invaginates into the more distal bowel. The pathomechanism is thought to involve altered bowel peristalsis at the intraluminal lesion, which is then a lead point for the intussusceptum. Inflammatory fibroid polyp (IFP) is a rare benign cause of intussusception.

Research frontiers

IFP is a benign gastrointestinal tumor that appears grossly as a localized, submucosal, sessile polypoid mass, occasionally involving the entire thickness of the gastrointestinal tract wall. In this study, the author performed an overall evaluation of the literature published in English language regarding the management approaches and diagnostic process in cases of intussusception related to IFPs and presented his own experience.

Innovations and breakthroughs

This study is the largest literature screening on intussusception due to IFPs.

Applications

This literature review demonstrates the importance of radiological assessment, surgical experience, and immunhistochemical staining in the differential diagnosis of IFPs.

Terminology

Intussusception is defined as invagination of a proximal part of the small bowel along with its mesentery into an adjacent segment; IFPs are rare, idiopathic pseudo tumorous lesions of the gastrointestinal tract, which were first described by Vanek in 1949 as an eosinophilic submucosal granuloma.

Peer review

The authors have prepared a literature review of 56 papers about IFP and intussusception published between 1976 and 2011 with a case that was treated in their clinic. Looking to the study in general, it seems simple and informative.

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BRIEF ARTICLE

Evaluation of sorafenib for hepatocellular carcinoma by contrast-enhanced ultrasonography: A pilot study

Kazue Shiozawa, Manabu Watanabe, Yoshinori Kikuchi, Takahide Kudo, Kenichi Maruyama, Yasukiyo Sumino

Kazue Shiozawa, Manabu Watanabe, Yoshinori Kikuchi, Yasukiyo Sumino, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Toho University Medical Center, Omori Hospital, Tokyo 143-8541, Japan
Takahide Kudo, Kenichi Maruyama, Department of Clinical Functional Physiology, Toho University Medical Center, Omori Hospital, Tokyo 143-8541, Japan
Author contributions: Shiozawa K, Watanabe M and Kikuchi Y designed the study; Kudo T and Maruyama K performed the examination; Shiozawa K and Watanabe M analyzed the data and wrote the manuscript; Sumino Y supervised the study.
Correspondence to: Manabu Watanabe, MD, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Toho University Medical Center, Omori Hospital, 6-11-1 Omorinishi, Ota-ku, Tokyo 143-8541, Japan. manabu62@med.toho-u.ac.jp
Telephone: +81-3-37624151 Fax: +81-3-37638542
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Abstract

AIM: To determine the usefulness of arrival time parametric imaging (AtPI) using contrast-enhanced ultrasonography (CEUS) with Sonazoid in evaluating early response to sorafenib for hepatocellular carcinoma (HCC).

METHODS: Fourteen advanced HCC patients who received sorafenib 400/800 mg/d for at least 4 wk and were followed up by CEUS were enrolled in this study. CEUS was performed before treatment and 2 and 4 wk after treatment, and images of the target lesion in the arterial phase were recorded for each patient. The images were analyzed by AtPI. Color mapping (CM) images obtained by AtPI were compared before and after the treatment. In these CM images, the mean arrival time of the contrast agent in the region of interest from the starting point [mean time (MT)] was calculated. In each patient, differences between MT before and MT 2 and 4 wk after the treatment were compared with responses evaluated 4-8 wk after the treatment by dynamic computed tomography (CT), and statistical analysis was performed. Modified response evaluation criteria in solid tumors was used for the response evaluation.

RESULTS: In CM images both 2 and 4 wk after the treatment, delays in the arrival time of the contrast agent were noted in 8 of the 14 patients. In the other 6 patients, no color changes were observed in the tumor, or red and/or yellow increase, suggesting a decrease in blood flow velocity between images 2 and 4 wk after the treatment and those before the treatment. Dynamic CT could be performed 4-8 wk after the treatment in 13 of the 14 patients. Median differences in the MT were 1.13 s and 1.015 s, 2 and 4 wk after the treatment, respectively, in the 8 patients who showed stable disease (SD)/partial response (PR) on dynamic CT. Median differences in the MT were -0.39 s and -0.95 s, 2 and 4 wk after the treatment, respectively, in the 5 patients who showed progressive disease (PD). Differences in the median MT between SD/PR and PD groups were significant 2 and 4 wk after the treatment with $P = 0.019$ and $P = 0.028$, respectively.

CONCLUSION: AtPI by CEUS using Sonazoid is suggested to be useful for evaluating early responses to sorafenib.

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Key words: Sorafenib; Sonazoid; Contrast-enhanced ultrasonography; Hepatocellular carcinoma; Therapeutic response

Peer reviewers: Herwig R Cerwenka, Professor, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, Graz A-8036, Austria; Bijan Eghtesad, Associate Professor, Department of General Surgery, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, United States; Markus Peck-Radosavljevic, Professor, Department of Gastro-

enterologie and Hepatologie, Medizinische Universität Wien, Vienna A-1090, Austria

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INTRODUCTION

We previously evaluated the hemodynamics of hepatocellular carcinoma (HCC) and metastatic liver tumors based on the time intensity curve (TIC)[1] and arrival time parametric imaging (AtPI)[2,3] employing contrast-enhanced ultrasonography (CEUS) using Sonazoid (Daiichi Sankyo, Tokyo, Japan). As a result, CEUS using Sonazoid has been suggested to allow continuous observation of hemodynamics and is applicable to evaluate the hemodynamics of HCC and the effects of therapeutics on metastatic liver tumors[1,2]. Recently, molecular biological characteristics related to the progression and proliferation of HCC have been clarified, accelerating the development of various molecular-targeting agents[4-6]. Sorafenib[7-9] (Bayer, Leverkusen, Germany) is a multikinase inhibitor that targets multiple molecules, and was approved for use in the treatment of unresectable advanced HCC in Japan in May, 2009. Since it targets tumor growth (RAF-MEK-ERK) and angiogenesis (vascular endothelial growth factor receptor and platelet-derived growth factor receptor) signal transduction pathways, it is expected to be useful for the treatment of HCC[10-14].

The evaluation of chemotherapeutic agents that exert their cytotoxic effects against advanced HCC by inhibiting nucleic acid metabolism has been conducted mainly based upon the tumor volume-reducing effect, according to the response evaluation criteria in solid tumor (RECIST)[15]. The tumor volume-reducing effect of molecular-targeting agents such as sorafenib appears to be slow, although the survival period is considered to be prolonged, even with only a minor volume-reducing effect. Indeed, in the Sorafenib HCC Assessment Randomized Protocol trial in advanced HCC, while the response rate based upon RECIST was only 2%, the survival period in the sorafenib group was significantly longer than in the placebo group, demonstrating its clinical efficacy[10]. Therefore, evaluation of the antineoplastic effect of sorafenib, which exhibits an antitumor effect primarily by inhibiting angiogenesis, is difficult by conventional criteria, and the evaluation of hemodynamics is expected to become important in the assessment of its efficacy[16]. Recently, the modified RECIST (mRECIST)[17] and Choi criteria[18] have been recommended for the evaluation of therapeutic effects[19]. Also, in consideration of the serious complications of sorafenib reported to date[10], and moreover the fact that sorafenib is an expensive drug, early evaluation of its therapeutic effects is considered to be necessary to assess whether the treatment should be continued.

In this study, we performed image analysis by AtPI using CEUS with Sonazoid before and after the sorafenib administration and evaluated the usefulness of AtPI in evaluating early responses to sorafenib.

MATERIALS AND METHODS

Of the 45 patients with advanced HCC in whom treatment with sorafenib was initiated at our hospital between June 2009 and October 2011, 14 who consented to the study and were orally treated with sorafenib for at least 4 wk, and who could be followed up by CEUS, were selected as subjects. All patients were males with a mean age of 70.4 years (62-82 years). Underlying liver disease was hepatitis C in 8, alcoholic hepatitis in 4, and others in 2. Child-Pugh liver function class was A in all subjects, median alpha-fetoprotein level before administration was 150.9 ng/mL (7.1-22 516 ng/mL), and median protein induced by vitamin K absence-Ⅱ level was 1781 mAU/mL (12-259 000 mAU/mL). The initial dose of sorafenib was 800 mg/d for 5 patients and 400 mg/d for 9 patients.

Methods

CEUS was performed before and 2 and 4 wk after the sorafenib administration. One lesion or portal vein tumor thrombus (PVTT) that could be followed for a period was selected on employing ultrasonography in each patient to standardize evaluations, and CEUS was performed in the same cross-section and under the same conditions at all time points. The ultrasound equipment used in this examination was SSA-790A (Toshiba Medical Systems, Tokyo, Japan) with a convex probe (PVT-375BT, 3.75-MHz center frequency). The imaging mode used was wideband harmonic imaging (pulse subtraction) with transmission/reception frequencies of 1.8 and 3.5 MHz, respectively. The mechanical index for acoustic output was set to 0.2; the dynamic range was set to 60-65 dB. A single focus point was set at the deep site of the lesion, and a bolus intravenous injection of Sonazoid (0.5 mL) was administered *via* a left cubital venous line followed by 10 mL normal saline flush. After injection of Sonazoid, the patients were asked to hold their breaths. The arterial phase (0-40 s) was observed and video images were recorded and analyzed by an off-line procedure using AtPI.

AtPI was performed using image analysis software for Aplio/Xario on the basis of the report by Watanabe *et al*[2]. It was performed by determining a starting point at an appropriate site such as an intrahepatic artery and a tumor vessel, regarding the time when the contrast agent reached this site as the zero point, measuring the difference in the arrival time between the target and starting points throughout the entire diagnostic image, and coloring the time differences [color mapping (CM)]. In this study, the moment of arrival of the contrast agent at a

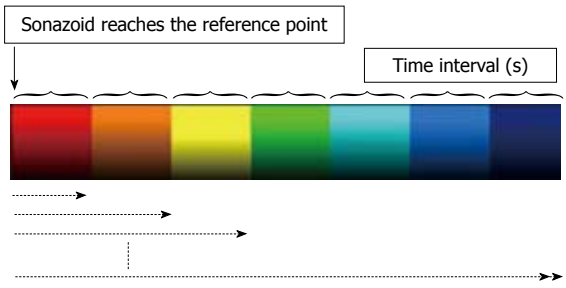


Figure 1 Delays in the arrival of the contrast agent at the target site compared with that at the reference point (0 s) are represented by red, orange, yellow, green, light blue, blue and dark blue at 0.5 s intervals.

large artery near the tumor or PVTT was regarded as starting point.

Qualitative analysis: Delays in the arrival of the contrast agent at the target site compared with that at the reference point (0 s) were represented by red→orange →yellow→green→light blue→blue→dark blue at 0.5 s intervals (Figure 1). CM images obtained were grossly compared in each patient before and after the treatment.

Quantitative analysis: In prepared CM images, a maximum region of interest (ROI) was determined for each subject, and the mean arrival time of the contrast agent in the ROI from the starting point [mean time (MT)] was calculated. In each patient, differences in the MT 2 and 4 wk after the initiation of the treatment compared with the MT before treatment were determined. Blood flow velocity was judged to have been reduced when the difference was zero or greater [MT (+) group] and to have been increased when the difference was less than zero [MT (-) group]. Differences in the MT 2 and 4 wk after the treatment were compared with responses evaluated 4-8 wk after the treatment by dynamic computed tomography (CT), and statistical analysis was performed. mRECIST[7] was used for the response evaluation. The Mann-Whitney test was performed for statistical analyses at the $P < 0.05$ level of significance. This study was approved by the Ethical Review Board of Toho University Medical Center, Omori Hospital.

RESULTS

In 14 patients, the mean duration of sorafenib administration was 177 d, and the mean daily dose was 542.9 mg/d.

Qualitative analysis

In CM images 2 wk after the treatment, color in the tumor changed to primarily blue or dark blue from primarily red or yellow before the treatment in 8 of the 14 patients, and time-dependent changes (delays in the arrival time of the contrast agent) were noted. These 8 patients also showed similar changes in CM images 4 wk after the treatment (Figure 2). In the other 6 patients, no color changes were observed in the tumor, or red and/or yellow increases, between images 2 wk after the treatment and those before the treatment. These 6 patients also showed similar CM images 4 wk after the treatment.

Quantitative analysis

The MT was (+) in 8 patients but (-) in 6 two weeks after the treatment, and mean differences in MT were 1.21 ± 0.75 s and -0.85 ± 0.78 s, respectively. Four weeks after the treatment, the MT was (+) in 7 and (-) in 7, and the mean difference in MT was 1.18 ± 0.4 s and -1.11 ± 0.62 s, respectively. The MT was (+) but changed to (-) from 2 to 4 wk after the treatment in 1 of the 8 patients in whom CM images showed gross delays in the arrival time of the contrast agent in the tumor 2 and 4 wk after the treatment.

Dynamic CT could be performed 4-8 wk after the treatment in 13 of the 14 patients. Median differences in the MT were 1.13 s and 1.015 s, 2 and 4 wk after the treatment, respectively, in the 8 patients who showed stable disease (SD)/partial response (PR) on dynamic CT. Median differences in the MT were -0.39 s and -0.95 s, 2 and 4 wk after the treatment, respectively, in the 5 patients who showed progressive disease (PD). Differences in the median MT between SD/PR and PD groups were significant 2 and 4 wk after the treatment with $P = 0.019$ and $P = 0.028$, respectively (Figure 3). The finding on dynamic CT after treatment was SD in the above patient in whom the MT changed from (+) to (-) 4 wk after the treatment.

DISCUSSION

Some studies have evaluated the responses of gastrointestinal stromal tumor (GIST) and metastatic lesions of renal cell carcinoma to molecular-targeted agents by CEUS using Levovist (Schering, Berlin, Germany) and SonoVue (Bracco, Milan, Italy)[20-24]. Lassau *et al*[20] evaluated the responses of GIST to imatinib by CEUS using Levovist and SonoVue, and suggested that CEUS was useful for detailed evaluation early after the treatment, and that decreases in tumor staining early after the treatment were related to progression-free survival (PFS) and may serve as a response-predicting factor. Lamuraglia *et al*[21] also evaluated the efficacy of sorafenib against liver metastases of renal cell carcinoma by CEUS using SonoVue, and suggested that PFS or overall survival (OS) may be prolonged by the suppression of vascularization.

In this study, we evaluated the early responses of advanced HCC to sorafenib by AtPI using CEUS with Sonazoid. Differences in the arrival time of the contrast agent between a large artery near the target lesion and ROI were evaluated according to CM images or the MT. Evaluations of differences in the arrival time are considered equivalent to evaluations of blood flow velocity to the tumor or inflow volume of blood flow in the tumor.

In this study, a delay in the arrival time of the contrast agent, i.e., a decrease in blood flow velocity, could be detected visually and readily in CM images as early as 2 wk after the treatment in 8 of the 14 patients. A decrease in blood flow velocity could also be confirmed

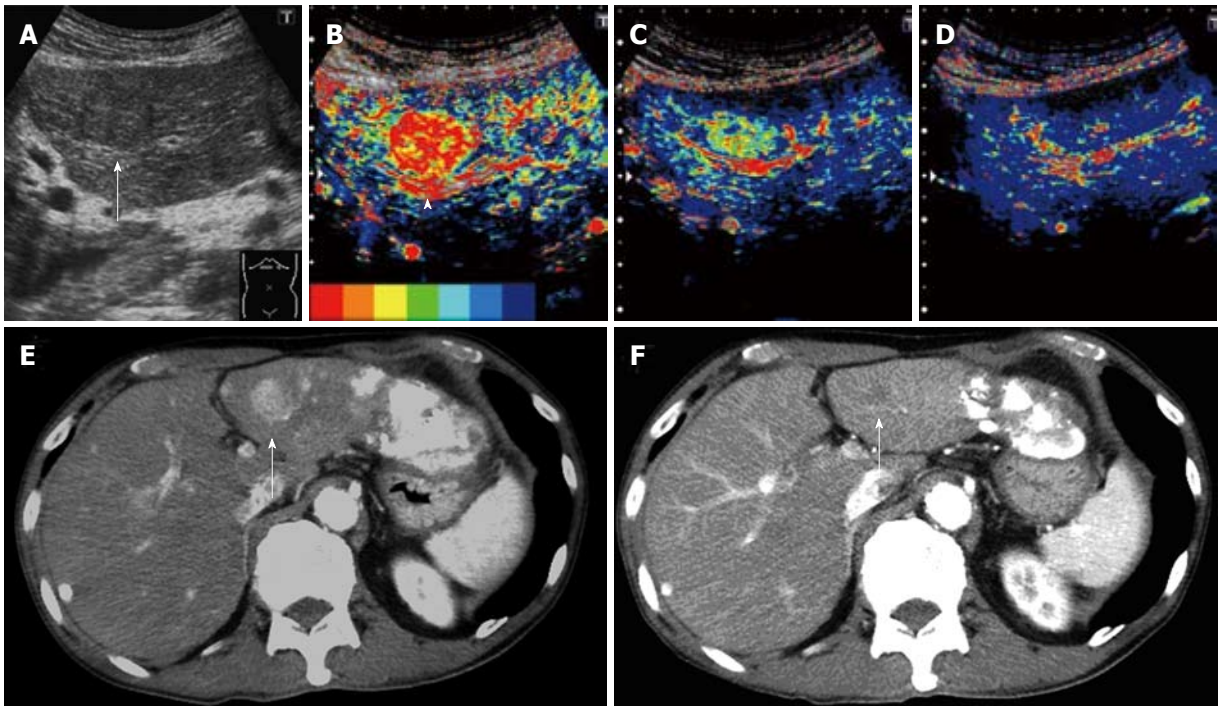


Figure 2 Clinical example of good responder patient. A 74-year-old man with a history of hepatitis C virus cirrhosis underwent transcatheter arterial chemoembolization (TACE) for advanced hepatocellular carcinoma three times, after which sorafenib administration (400 mg/d) was started for this patient because the tumor was TACE-refractory. A: Gray-scale ultrasonography showed a mosaic-pattern tumor sized 20 mm in diameter in S3 (arrow). This tumor was established as a target lesion; B: A large artery near this tumor was regarded as starting point (arrow head). The color mapping (CM) image before the treatment showed primarily red or orange in the tumor; C: The CM image 2 wk after the treatment showed primarily green in the tumor; D: The CM image 4 wk after the treatment showed primarily dark blue in the tumor, the same as the surrounding parenchyma; E: Dynamic computed tomography (CT) scan in arterial phase before the treatment showed a hypervascular lesion in S3 (arrow) which was the target lesion and accumulation of iodized oil in the left lobe and S7; F: Dynamic CT scan in arterial phase 4 wk after the treatment showed a hypovascular lesion in S3 (arrow) and this lesion reduced. This therapeutic response was described as a partial response.

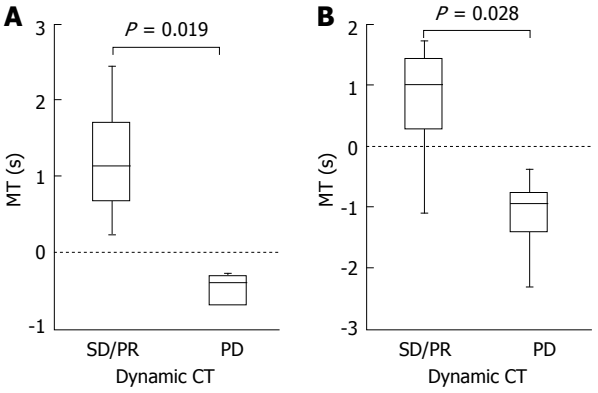


Figure 3 Comparisons between the mean time and therapeutic effects according to modified response evaluation criteria in solid tumors evaluated by dynamic computed tomography. A: Median difference in the mean time (MT) was 1.13 s, 2 wk after the treatment in the 8 patients who showed stable disease (SD)/partial response (PR) on dynamic computed tomography (CT). Median difference in the MT was -0.39 s, 2 wk after the treatment in the 5 patients who showed progressive disease (PD); B: Median difference in the MT was 1.015 s, 4 wk after the treatment, in the 8 patients who showed SD/PR on dynamic CT. Median difference in the MT was -0.95 s 4 wk after the treatment in the 5 patients who showed PD. Differences in the median MT between SD/PR and PD groups were significant 2 and 4 wk after the treatment with $P = 0.019$ and $P = 0.028$, respectively.

after 4 wk. However, as changes in blood flow in the tumor due to sorafenib administration were uneven, we set a ROI in the target HCC, calculated the mean arrival time of the contrast agent into the ROI, i.e., MT, and evaluated differences in the MT before and after the treatment as an objective method, not dependent on vision. In the 8 patients in whom delays in the arrival time of the contrast agent could be confirmed visually in CM images both 2 and 4 wk after the treatment, the MT was (+) in all 8 after 2 wk and in 7 after 4 wk. This suggests the possibility of quantification and objective evaluation of changes in blood flow velocity in the tumor visually detected in CM images. Comparisons between the MT and therapeutic effects according to mRECIST evaluated by dynamic CT showed significant differences in the MT between SD/PR and PD groups both 2 and 4 wk after the treatment, indicating general agreement between these parameters. The results of this study suggest that a delay in the arrival time of the contrast agent visually detected in CM images, i.e., a change of CM images, reflect the response and that changes in hemodynamics visually represented in CM images may be quantified and objectively evaluated using the MT.

Responses of HCC have been evaluated primarily using dynamic CT and magnetic resonance imaging as well as CEUS, but it has often been difficult to repeat the examination frequently during the course due to exposure, iodine allergies, and renal dysfunction. On the other hand, Sonazoid is effective at a low dose, causes few adverse reactions, and can be used safely even in patients with iodine allergies or renal dysfunction, with the

exception of those with egg shell allergies. Since changes in CM images were noted in this study, and the MT showed differences from values before the treatment by 2 wk after the treatment, AtPI using CEUS with Sonazoid may be useful for the early evaluation of responses to sorafenib.

Lassau *et al*[22] reported that evaluation of the time to peak intensity and slope of the wash-in obtained by TIC using CEUS with SonoVue was useful for the predication of early responses of metastatic lesions of renal cell carcinoma to molecular-targeted agents. These techniques including AtPI reflect tumor hemodynamics, and their relative usefulness is impossible to discuss, but AtPI may be equally predictive of responses compared to the TIC.

This study raises the following questions: (1) Since tumors have a three-dimensional structure, can they be evaluated accurately by examination of a single cross-sectional ultrasonography image? (2) Since HCC shows multicentric carcinogenesis[25,26] and intrahepatic metastasis, the degree of differentiation may vary among lesions of multiple HCC. Therefore, is it possible to apply the findings in a single target lesion uniformly to all other lesions? (3) Visual evaluation of CM images obtained 4 wk after the treatment disagreed with the MT value in 1 patient. While CM images were found to be useful for simple comparison between conditions before and after the treatment, measurement of the MT is still a complex process, and further improvements are considered necessary in the method to determine the ROI and other aspects; and (4) Do the results obtained by this study, i.e., the MT calculated by AtPI, contribute to improvements in the OS? These problems are considered to need careful evaluation by further accumulation of cases.

In conclusion, AtPI by CEUS using Sonazoid is suggested to be useful for evaluating early responses to sorafenib. Changes in hemodynamics in advanced HCC over the course of its treatment could be visually represented in a single static CM image obtained by AtPI. By further quantification, hemodynamic changes over the course of treatment could be evaluated more objectively. Since the number of patients in this study was small, and the observation period was short, the usefulness of the procedure in evaluating the time to progression or OS cannot be discussed, but evaluation of tumor hemodynamics using AtPI is considered to contribute to the prediction of responses early after treatment.

COMMENTS

Background

Recently, molecular biological characteristics related to the progression and proliferation of hepatocellular carcinoma (HCC) have been clarified, accelerating the development of various molecular-targeted agents. Since sorafenib is a multikinase inhibitor and it targets tumor growth and angiogenesis signal transduction pathways, it is expected to be useful for the treatment of HCC.

Research frontiers

Therapeutic efficacy has been assessed by Response Evaluation Criteria in Solid Tumor using dynamic computed tomography (CT) and overall survival in previous studies. Since sorafenib produces an antitumor effect primarily by inhibiting angiogenesis, evaluation of its antineoplastic effect is difficult by conventional criteria, and evaluating hemodynamics is expected to become important for its efficacy evaluation. In this study, the authors verified that arrival time parametric imaging (AtPI) by contrast-enhanced ultrasonography (CEUS) using Sonazoid is useful for evaluating early responses to sorafenib.

Innovations and breakthroughs

Dynamic CT and magnetic resonance imaging are generally used to evaluate the antineoplastic effect, but tumor hemodynamics are difficult to evaluate by these methods. AtPI by CEUS can evaluate the changes of hemodynamics in HCC over a course of treatment visually and objectively.

Applications

AtPI by CEUS using Sonazoid is suggested to be useful for evaluating early responses to sorafenib. This was a pilot study with a small series of patients. Similar studies with much larger numbers of patients are awaited.

Terminology

Ultrasound contrast agents consist of microbubbles that visualize the hemodynamics. AtPI is a method using CEUS, which utilizes the arrival time of each pixel in the diagnostic image.

Peer review

This is an interesting manuscript on a small series of patients followed with CEUS under treatment with sorafenib. As a pilot, it provides an interesting hypothesis to be tested in a larger series of patients. It is well conducted and also well presented.

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BRIEF ARTICLE

Nutritional status in relation to lifestyle in patients with compensated viral cirrhosis

Fumikazu Hayashi, Chika Momoki, Miho Yuikawa, Yuko Simotani, Etsushi Kawamura, Atsushi Hagihara, Hideki Fujii, Sawako Kobayashi, Shuji Iwai, Hiroyasu Morikawa, Masaru Enomoto, Akihiro Tamori, Norifumi Kawada, Satoko Ohfuji, Wakaba Fukusima, Daiki Habu

Fumikazu Hayashi, Chika Momoki, Miho Yuikawa, Yuko Simotani, Daiki Habu, Department of Medical Nutrition, Graduate School of Life Science, Osaka City University, Osaka 558-8585, Japan
Etsushi Kawamura, Atsushi Hagihara, Hideki Fujii, Sawako Kobayashi, Shuji Iwai, Hiroyasu Morikawa, Masaru Enomoto, Akihiro Tamori, Norifumi Kawada, Department of Hepatology, Graduate School of Medicine, Osaka City University, Osaka 545-8585, Japan
Satoko Ohfuji, Wakaba Fukusima, Department of Public Health, Graduate School of Medicine, Osaka City University, Osaka 545-8585, Japan
Author contributions: Hayashi F performed the research and wrote the manuscript; Yuikawa M and Simotani Y performed the research; Kawamura E, Hagihara A, Fujii H, Kobayashi S, Iwai S, Morikawa H, Enomoto M, Tamori A and Kawada N introduced the patients to the study and received the agreements of all participating patients; Momoki C, Ohfuji S and Fukusima W supported the study and performed statistical analysis; Habu D designed the study.
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Correspondence to: Daiki Habu, MD, Professor of Life Science, Department of Medical Nutrition, Graduate School of Life Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan. habu@life.osaka-cu.ac.jp
Telephone: +81-6-66052892 Fax: +81-6-66052892
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Abstract

AIM: To assess the nourishment status and lifestyle of non-hospitalized patients with compensated cirrhosis by using noninvasive methods.

METHODS: The subjects for this study consisted of 27 healthy volunteers, 59 patients with chronic viral hepatitis, and 74 patients with viral cirrhosis, from urban areas. We assessed the biochemical blood tests, anthropometric parameters, diet, lifestyle and physical activity of the patients. A homeostasis model assessment-insulin resistance (HOMA-IR) value of $\geqslant$ 2.5 was considered to indicate insulin resistance. We measured height, weight, waist circumference, arm circumference, triceps skin-fold thickness, and handgrip strength, and calculated body mass index, arm muscle circumference (AMC), and arm muscle area (AMA). We interviewed the subjects about their dietary habits and lifestyle using health assessment computer software. We surveyed daily physical activity using a pedometer. Univariate and multivariate logistic regression modeling were used to identify the relevant factors for insulin resistance.

RESULTS: The rate of patients with HOMA-IR $\geqslant$ 2.5 (which was considered to indicate insulin resistance) was 14 (35.9%) in the chronic hepatitis and 17 (37.8%) in the cirrhotic patients. AMC (%) (control *vs* chronic hepatitis, 111.9% ± 10.5% *vs* 104.9% ± 10.7%, P = 0.021; control *vs* cirrhosis, 111.9% ± 10.5% *vs* 102.7% ± 10.8%, P = 0.001) and AMA (%) (control *vs* chronic hepatitis, 128.2% ± 25.1% *vs* 112.2% ± 22.9%, P = 0.013; control *vs* cirrhosis, 128.2% ± 25.1% *vs* 107.5% ± 22.5%, P = 0.001) in patients with chronic hepatitis and liver cirrhosis were significantly lower than in the control subjects. Handgrip strength (%) in the cirrhosis group was significantly lower than in the controls (control *vs* cirrhosis, 92.1% ± 16.2% *vs* 66.9% ± 17.6%, P < 0.001). The results might reflect a decrease in muscle mass. The total nutrition intake and amounts of carbohydrates, protein and fat were not significantly different amongst the groups. Physical activity levels (kcal/d) (control *vs* cirrhosis, 210 ± 113 kcal/d *vs* 125 ± 74 kcal/d, P = 0.001), number of steps (step/d) (control *vs* cirrhosis, 8070 ±

3027 step/d *vs* 5789 ± 3368 step/d, P = 0.011), and exercise (Ex) (Ex/wk) (control *vs* cirrhosis, 12.4 ± 9.3 Ex/wk *vs* 7.0 ± 7.7 Ex/wk, P = 0.013) in the cirrhosis group was significantly lower than the control group. The results indicate that the physical activity level of the chronic hepatitis and cirrhosis groups were low. Univariate and multivariate logistic regression modeling suggested that Ex was associated with insulin resistance (odds ratio, 6.809; 95% CI, 1.288-36.001; P = 0.024). The results seem to point towards decreased physical activity being a relevant factor for insulin resistance.

CONCLUSION: Non-hospitalized cirrhotic patients may need to maintain an adequate dietary intake and receive lifestyle guidance to increase their physical activity levels.

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Key words: Hepatitis C virus; Insulin resistance; Liver cirrhosis; Nutrition assessment; Obesity; Lifestyle; Exercise

Peer reviewers: Dr. Laura E Matarese, Department of Gastroenterology, Hepatology and Nutrition, East Carolina University, 600 Moye Blvd., Greenville, NC 27834, United States; Takumi Kawaguchi, MD, PhD, School of Medicine, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan

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INTRODUCTION

Chronic infection with hepatitis virus is one of the most common causes of liver cirrhosis worldwide. About 3.5 million people worldwide are chronically infected with hepatitis B virus (HBV). More than 70% of all newly diagnosed liver cancer patients are found in Asia, a region accounting for 75% of all patients with chronic HBV infection[1]. Hepatitis C virus (HCV) has infected approximately 1.3 million people worldwide. In the Asia-Pacific region, the prevalence of HCV infection ranges from 0.3% to 12%, with geographical variation[2]. The major cause of liver cirrhosis in Japan is the hepatitis virus. In particular, chronic HCV infection accounts for 60.9% of all liver cirrhosis cases[3].

Cirrhosis is an end-stage chronic liver disease, and the damage to the hepatocytes decreases the function of the liver, leading to a variety of nutritional metabolic disorders. Therefore, protein energy malnutrition (PEM) is frequently seen in patients with cirrhosis[4-7]. A study of subjects with cirrhosis conducted in Western countries reported that 11% had energy malnutrition, 22% had protein malnutrition, 32% had both complications of PEM, and 35% had a normal nourishment status[8]. In 2002, a study of Japanese patients with cirrhosis reported that 12% had energy malnutrition, 25% had protein malnutrition, 50% had PEM, and only 13% had a normal nourishment status[9]. Currently, the lifestyles of people in urban areas of several Asian countries are changing due to westernization and motorization, resulting in excessive food intake, decreased intensity of daily activities, and a lack of exercise (Ex). These changes may lead to obesity, metabolic syndrome, and lifestyle diseases. Is there a possibility that this lifestyle transition (over-nourishment and decline in the intensity of daily activities) has an effect on the nutrition of cirrhosis? In 2006, a study reported that body mass index (BMI) in patients with cirrhosis was almost the same as those of the same age group in the normal Japanese population[10]. Moreover, both experimental and clinical studies found that over-nutrition, fatty liver, and glucose intolerance are significant risk factors of hepatic carcinogenesis[11,12]. The combination of over-nutrition and obesity in cirrhosis patients also indicates the possibility of an impaired pathogenesis. These findings suggest that high calorie and protein intake (i.e., old dietary interventions of cirrhosis) promotes obesity, fatty liver, and hepatic carcinogenesis. Almost all subjects in Japanese reports were hospitalized having decompensated cirrhosis with hepatic failure. Patients with severe decompensated cirrhosis tend to develop a reduced food and protein intake because of abdominal ascites and hepatic encephalopathy. However, the majority of cirrhotic patients have compensated cirrhosis and so do not need to be hospitalized, and thus their daily lives may be almost the same as that of healthy people. Few researchers have studied the effect of dietary habits and lifestyle in non-hospitalized cirrhotic patients on their conditions.

If we consider cirrhosis from the point of view of a lifestyle disease, we should know the patients' actual condition in order to provide adequate lifestyle and dietary guidance. The purpose of this study is to assess the nourishment status of non-hospitalized patients with compensated cirrhosis and to create a database on nutritional and lifestyle education for these patients by analyzing their lifestyle, food intake and physical activity.

MATERIALS AND METHODS

Subjects

We interviewed 155 patients (age range: 45-79 years) with chronic liver disease living in urban areas. Patients with chronic viral liver disease who regularly visited the Osaka City University Hospital between March 2009 and December 2010 were enrolled. Written consent was received from all study participants. After excluding patients with non-viral liver disease, abdominal ascites and hepatic encephalopathy, 133 patients were enrolled. Next, the

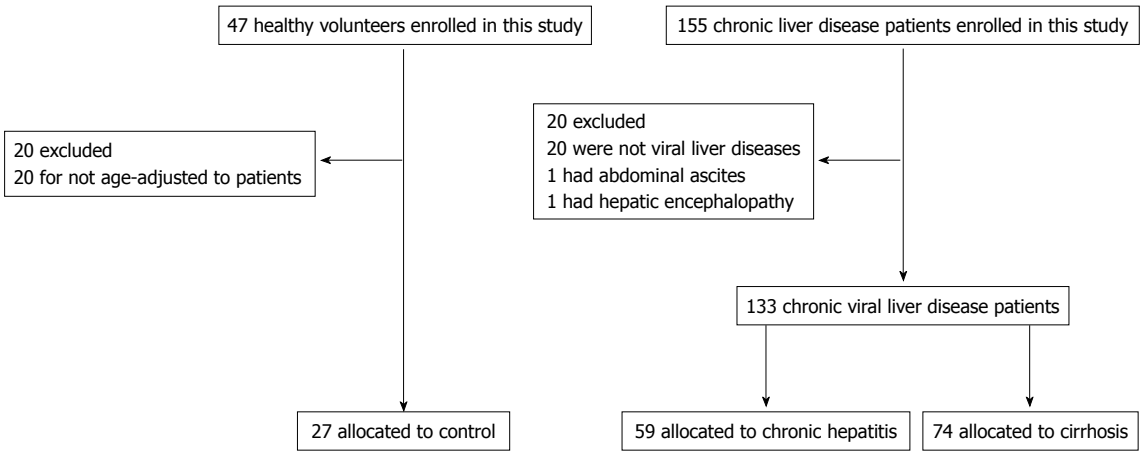


Figure 1 Flow chart of the participants in the study.

patients were divided into 2 groups of chronic hepatitis and cirrhosis on the basis of the clinical diagnosis made by the attending physicians. We enrolled 47 healthy volunteers from an urban area (local residents around the Osaka City University) who underwent a health check after providing their consent. The results were age-adjusted and, finally, data of 27 healthy volunteers were used for the comparison of the subjects (Figure 1). Complication of diabetes mellitus, hypertension, and hyperlipidemia were defined by taking medication for each disease. The degree of liver disease was assessed using the Child-Pugh classification. This trial was conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki (1996) and was approved by the ethics committee of our institution (No. 1906).

Laboratory evaluation

Both chronic hepatitis and cirrhosis patients were examined, and we measured the number of white blood cells (WBC; × 100/μL), number of red blood cells (RBC; × 10^2/μL), hemoglobin (Hb; g/dL), hematocrit level (Ht; %), platelet count (Plt; × 10^2/μL), prothrombin time (PT; %); total protein (TP; g/dL), albumin (Alb; g/dL), blood urea nitrogen (mg/dL), creatinine (mg/dL), total bilirubin (T-bil; mg/dL), direct bilirubin (D-bil; mg/dL), aspartate aminotransferase (AST; IU/L), alanine aminotransferase (IU/L), leucine aminopeptidase (LAP; IU/L), γ-glutamyl transpeptidase (IU/L), alkaline phosphatase (ALP; IU/L), cholinesterase (ChE; IU/L), triglycerides (TG; mg/dL), total cholesterol (Total-C; mg/dL), fasting blood sugar (FBS; mg/dL), hemoglobin A1c (HbA1c; %), immunoreactive insulin levels (IRI; μU/mL), plasma branched-chain amino acid levels (BCAA; μmol/mL), plasma tyrosine levels (Tyrosine; μmol/mL), and the molar ratio of BCAA to tyrosine (BTR). The insulin resistance index was calculated according to homeostasis model assessment-insulin resistance (HOMA-IR) from the FBS and IRI values using the following formula[13]: HOMA-IR = FBS × IRI/405.

A HOMA-IR value of ⩾ 2.5 was considered to indicate insulin resistance. The data of subjects with complicated diabetes mellitus were excluded in the comparison of the HOMA-IR values.

Anthropometric assessment

To evaluate nourishment status in the subjects, we assessed their height (cm) and weight (kg) and calculated BMI (kg/m^2). We classified patients with a BMI of < 18.5 kg/m^2 as underweight, 18.5 kg/m^2 to 25 kg/m^2 as normal, and ⩾ 25 kg/m^2 as obese to estimate the rate of obesity in the subjects[14]. We measured the waist circumference (cm) by using a measuring tape to estimate the intra-abdominal fat. Arm circumference (AC; cm) and triceps skin-fold thickness (TSF; mm) were measured with Inser-Tape and Adipometer (Abbott Japan Co., Ltd., Tokyo, Japan). To minimize intra-operator variability, the averages of 3 consecutive measurements were recorded. Arm muscle circumference (AMC; cm) and arm muscle area (AMA; cm^2) were calculated by using the following formula: AMC = AC - (π × TSF), AMA = (AMC)^2/4π.

Age and sex difference were considered; the mean value of measured anthropometric measurements were divided by the respective age-sex-adjusted medians, which were obtained from the Japanese Anthropometric Reference Data 2001[15]. The results were expressed as a percentage (%TSF, %AC, %AMC, %AMA). In addition, right and left handgrip strength were measured once by using the Smedley Type Hand Dynamometer Grip D (Takei Kiki Kogyo Co., Ltd., Japan). The mean value of both hand grip strength measurements were expressed as a percentage by dividing the average of each age acquired from the Japanese Ministry of Education, Culture, Sports, Science and Technology, which performed a test of physical strength and Ex capacity in 2009. The obtained results were used as a qualitative evaluation index of the skeletal muscle.

Dietary and lifestyle survey

Dietary habits and lifestyle of the interviewed subjects were evaluated by using the specific health guideline support software Health Check for Metabolic Syndrome (Su-

zuken Co., Ltd., Nagoya, Japan). Originally, this software was developed as a tool for specific health guidelines to study metabolic syndromes and reserves. The software was modified so that it could be used for chronic liver disease patients. The survey consisted of 50 questions concerning daily life and dietary habits. The results were expressed as total energy or protein intake per body (BW). To compare the results of this study with the guideline of the European Society for Parenteral and Enteral Nutrition (ESPEN) Nutritional Management[16], total energy and protein intake were divided by BW. The results were expressed as total energy or protein intake per BW.

Physical activity survey

The lifestyle recorder, Lifecorder (Suzuken Co., Ltd., Nagoya, Japan), is an activity observation machine that functions as an acceleration sensor. The physical activity pattern was objectively obtained by continuously measuring activity intensity, period and frequency. This is one means to evaluate physical activity-related energy expenditure. It is possible to estimate total energy expenditure (TEE), including involuntary activities such as slow or small erratic movements performed when sedentary and during very low-level activities, by attaching the Lifecorder for 24 h. The quantity and intensity of physical activity could then be accurately determined. This was confirmed in a paper that reported the use of the metabolic chamber[17], showing correlation with the measured values of TEE. The Lifecorder was used for 1 wk (7 d). Data of daily TEE (kcal/d), physical activity (kcal/d), the number of steps (step/d), and Ex (Ex/wk) were obtained. Ex is a unit to express the quantity of physical activity, calculated by multiplying metabolic equivalents (METs) by the duration of the activity (h) as proposed in the Physical Activity Guide for Health Promotion 2006, which was published by the Ministry of Health, Labor and Welfare of Japan. The METs is an index for the intensity of physical activity, calculated as energy expenditure (oxygen uptake, mL/kg per minute) during a specific physical activity divided by sitting/ resting energy expenditure (REE). The goal for the quantity of physical activity for health promotion was set at 23 Ex or more per week to be achieved by active physical activities (including both Ex and non-Ex activities), of which 4 Ex or more should be achieved by active Ex[18].

Logistic regression analysis

Univariate and multivariate logistic regression modeling were used to identify the relevant factors for insulin resistance in patients with chronic liver disease. The degree of chronic hepatitis, HCV infection, the presence or absence of hepatocellular carcinoma, sex, age, %AC, %TSF, handgrip strength, Ex, Plt, Alb, BTR, total energy intake per BW, protein intake per BW, and the percentage fat in nutrition intake were chosen as potential factors. Continuous variables were divided into 2 groups according to each standard as described below. HCV infection has been reported to cause insulin resistance[19]. Therefore, patients were categorized into 2 groups according to HBV and HCV infection. Patients were categorized into 2 groups by age, with those over 65 years considered elderly. Both %AC and %TSF were divided using 100 as a standard value. Handgrip strength was divided using 69, which was the median value. Ex was divided using a value of 4.0, which is the desired amount of active Ex. Plt was divided according to a value of 100 000, with less than 100 000 as the standard for liver cirrhosis. It has been reported that Alb of 3.5 or less is related to prognosis[9]. Therefore, Alb was divided using a value of 3.5, with less than 3.5 considered malnutrition. BTR was divided into 2 groups based on 4.41, which is the lower limit of the standard range. In this study, energy intake per BW was divided based on a value of 30, which is the median of the standard range in the ESPEN nutritional management guidelines[16]. Protein intake per BW was divided based on a value of 1.2, which is the upper limit of the standard range in the ESPEN nutritional management guidelines. The percentage of fat in daily food intake was divided using 25, which is the upper limit of the standard range.

Statistical analysis

Outcomes are presented as mean ± SD, number of patients, or a percentage. The statistical analyses were performed using the Mann-Whitney *U*, Kruskal-Wallis, and Scheffe's multiple comparison tests, or Spearman's rank correlation coefficient. Univariate and multivariate logistic regression modeling were used to obtain the crude and adjusted odds ratios and 95% CI for the association between each factor and insulin resistance. The statistical software Dr. SPSS Ⅱ for Windows Version 11.0.1J (SPSS Inc.) was used for the analysis. P value < 0.05 was considered statistically significant.

RESULTS

Subjects characteristics

One hundred and thirty-three patients with chronic liver disease were included in the analysis. Their characteristics are summarized in Table 1. Sex was not significantly different among the groups. Age in cirrhotic patients was significantly higher than that in patients with chronic hepatitis. Seventeen patients with chronic hepatitis and 11 patients with cirrhosis were infected with HBV, while 42 patients with chronic hepatitis and 63 patients with cirrhosis were infected with HCV. Eleven patients had diabetes mellitus, 20 had hypertension, and 4 had hyperlipidemia in the chronic hepatitis patients. The cirrhotic patients were involved with complications of diabetes mellitus in 11 patients, hypertension in 20, and hyperlipidemia in 4. When cirrhotic patients were assessed by using Child-Pugh classification of the severity of liver disease, 62 patients were classified into class A, and 12 into class B.

Laboratory evaluation

The results of laboratory evaluation are shown in Table

Table 1 Subjects characteristics

	Control	Chronic hepatitis	Cirrhosis
Sex (male/female)	12/15	26/33	25/49
Age (yr)	65.9 ± 6.1	63.5 ± 10.1	68.3 ± 7.5[b]
Viral infection (HBV/HCV)	-	17/42	11/63
Child-Pugh classification (A/B)	-	-	62/12
Diabetes mellitus	-	11	11
Hypertension	-	20	24
Hyperlipidemia	-	4	6

[b]$P < 0.01$ *vs* chronic hepatitis. Data are expressed as mean ± SD or as number of patients. Statistical differences among the 3 groups were determined by the Kruskal-Wallis and Scheffe's multiple comparison tests. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 3 Anthropometric assessments of all subjects

	Control	Chronic hepatitis	Cirrhosis
Height (cm)[1]			
Male	168.2 ± 6.3	168.0 ± 7.6	165.8 ± 6.0
Female	151.0 ± 3.5	152.8 ± 4.1	151.6 ± 6.5
Weight (kg)[1]			
Male	67.0 ± 8.5	67.5 ± 11.0	64.5 ± 9.0
Female	54.5 ± 6.7	54.6 ± 9.4	50.8 ± 8.8
BMI (kg/m^2)[1]			
Male	23.7 ± 3.1	23.9 ± 3.1	23.4 ± 2.6
Female	23.9 ± 2.6	23.4 ± 4.0	22.1 ± 3.5
BMI (underweight/normal/obese)[2]			
Male	1/5/5	1/17/9	0/16/8
Female	1/8/7	2/17/13	6/36/8[h]
Abdominal circumference (cm)[1]			
Male	86.3 ± 7.8	90.0 ± 8.0	87.7 ± 8.6
Female	84.6 ± 10.0	86.0 ± 9.9	85.1 ± 9.2
%AC[1]	108.1 ± 8.4	107.4 ± 11.8	103.9 ± 11.5
%TSF[1]	96.0 ± 37.0[a]	129.0 ± 50.2	116.5 ± 48.4
%AMC[1]	111.9 ± 10.5[b,e]	104.9 ± 10.7	102.7 ± 10.8
%AMA[1]	128.2 ± 25.1[c,f]	112.2 ± 22.9	107.5 ± 22.5
Hand grip strength (%)[1]	92.1 ± 16.2[d,g]	74.4 ± 20.9	66.9 ± 17.6

[a]$P = 0.013$, [b]$P = 0.021$, [c]$P = 0.013$, [d]$P < 0.001$ *vs* chronic hepatitis; [e]$P = 0.001$, [f]$P = 0.001$, [g]$P < 0.001$ *vs* liver cirrhosis; [h]$P = 0.007$. Data are expressed as mean ± SD or as number of patients. [1,2]Statistical differences among the 3 groups were determined by the Kruskal-Wallis and Scheffe's multiple comparison tests[1] or Spearman's rank correlation coefficient[2]. BMI: Body mass index; AC: Arm circumference; TSF: Triceps skin fold thickness; AMC: Arm muscle circumference; AMA: Arm muscle area.

2. The values of WBC, RBC, Hb, Ht, Plt, PT, TP, Alb, Total-C, TG, ChE, HbA1c and BTR in cirrhotic patients were significantly lower than those in patients with chronic hepatitis. Furthermore, the values of AST, T-bil, D-bil, ALP and LAP were also significantly higher in the latter group. The IRI of both the groups was slightly high, and the mean value of HOMA-IR was ⩾ 2.5. Twenty-two of the 106 patients who had obvious diabetes, based on their measured FBS level and insulin level, were excluded. We used the remaining 84 patients as the subjects for the analysis. The number of patients with a HOMA-IR ⩾ 2.5, which was considered to indicate insulin resistance, was 14 (35.9%) in the chronic hepatitis patients and 17 (37.8%) in the cirrhotic patients.

Table 2 Laboratory evaluations for the patients (mean ± SD)

	Chronic hepatitis	Cirrhosis
WBC (× 100/μL)	55.5 ± 19.2	37.9 ± 13.6[a]
RBC (× 10^2/μL)	441 ± 57	399 ± 47[a]
Hb (g/dL)	13.8 ± 1.5	12.6 ± 1.6[a]
Ht (%)	40.7 ± 3.9	37.2 ± 4.0[a]
Plt (× 10^2/μL)	17.8 ± 3.8	8.6 ± 2.5[a]
PT (%)	96.9 ± 11.2	83.4 ± 13.2[a]
TP (g/dL)	7.2 ± 0.5	7.0 ± 0.7[b]
Alb (g/dL)	4.0 ± 0.4	3.5 ± 0.5[a]
BUN (mg/dL)	15.6 ± 6.1	15.5 ± 5.6
Cre (mg/dL)	0.77 ± 0.27	0.73 ± 0.24
T-bil (mg/dL)	0.9 ± 0.4	1.1 ± 0.5[c]
D-bil (mg/dL)	0.3 ± 0.2	0.4 ± 0.3[a]
AST (IU/L)	44 ± 23	60 ± 39[d]
ALT (IU/L)	41 ± 27	53 ± 40
AST/ALT	1.2 ± 0.4	1.3 ± 0.5
LAP (IU/L)	59 ± 20	63 ± 17[e]
γ-GTP (IU/L)	51 ± 85	51 ± 57
ALP (IU/L)	299 ± 144	356 ± 126[f]
ChE (IU/L)	281 ± 89	201 ± 81[a]
TG (mg/dL)	102 ± 44	87 ± 31
T-cho (mg/dL)	182 ± 37	155 ± 38[a]
HbA1c (%)	5.9 ± 0.8	5.2 ± 0.9[a]
FBS (mg/dL)	99.0 ± 14.1	99.6 ± 14.5
IRI (μU/mL)	11.1 ± 8.2	12.1 ± 11.1
HOMA-IR	2.7 ± 2.2	2.9 ± 2.5
BTR	5.43 ± 1.54	3.90 ± 1.23[a]
BCAA (μmol/mL)	425 ± 92	387 ± 77[g]
Tyrosine (μmol/mL)	82 ± 23	107 ± 36[a]

[a]$P < 0.05$, [b]$P = 0.018$, [c]$P = 0.017$, [d]$P = 0.008$, [e]$P = 0.048$, [f]$P = 0.001$, [g]$P = 0.025$ *vs* chronic hepatitis. Differences between the 2 groups were analyzed using the Mann-Whitney *U* test. WBC: Number of white blood cells; RBC: Number of red blood cells; Hb: Hemoglobin; Ht: Hematocrit level; Plt: Platelet count; PT: Prothrombin time; TP: Total protein; Alb: Albumin; BUN: Blood urea nitrogen; Cre: Creatinine; T-bil: Total bilirubin; D-bil: Direct bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LAP: Leucine aminopeptidase; γ-GTP: γ-glutamyl transpeptidase; ALP: Alkaline phosphatase; ChE: Cholinesterase; TG: Triglycerides; Total-C: Total cholesterol; HbA1c: Hemoglobin A1c; FBS: Fasting blood sugar; IRI: Immunoreactive insulin levels; HOMA-IR: Homeostasis model assessment-insulin resistance; BCAA: Plasma branched-chain amino acid levels; BTR: Molar ratio of BCAA to tyrosine; Tyrosine: Plasma tyrosine levels.

Anthropometric assessment

Table 3 presents the outcomes of anthropometric assessments in the subjects. The skeletal muscle index (measured as %AMC and %AMA) was significantly lower in the chronic hepatitis and cirrhotic patients than in the controls. The body fat indicator (measured as %TSF) in the chronic hepatitis patients was significantly higher than in the controls. The handgrip strength in both the patient groups was significantly lower than that in the controls. We divided the subjects into male and female subgroups when comparing height, weight, BMI and abdominal circumference. There were no significant differences among the 3 groups. Among the men, the rate of obesity was 45.5% in the control, 33.3% in those with chronic hepatitis, and 33.3% in those with liver cirrhosis. Among the women, the rate of obesity was 43.8% in the control, 40.6% in those with chronic hepatitis, and 16.0% in those with liver cirrhosis. The difference in the obesity rate was not significant in the men; however, the obesity rate in

Table 4 The dietary surveys of all subjects (mean ± SD)

	Control	Chronic hepatitis	Cirrhosis
Energy intake (kcal)	1814 ± 400	1768 ± 470	1730 ± 468
Energy intake (kcal/BW)	31.0 ± 7.5	30.0 ± 8.6	32.0 ± 9.0
Carbohydrate percentage (%)	57.9 ± 7.1	59.1 ± 9.1	60.1 ± 8.9
Protein percentage (%)	15.9 ± 2.9	16.2 ± 2.8	16.2 ± 3.0
Fat percentage (%)	26.1 ± 7.1	24.8 ± 8.4	23.7 ± 8.8
Carbohydrate intake (g)	248.9 ± 57.4	254.9 ± 76.5	251.2 ± 67.9
Protein intake (g)	68.0 ± 17.1	68.3 ± 17.1	70.0 ± 19.5
Protein intake (g/BW)	1.2 ± 0.3	1.2 ± 0.4	1.3 ± 0.4
Fat intake (g)	50.5 ± 19.5	47.4 ± 21.4	46.7 ± 24.4
Calcium intake (mg)	544 ± 198	513 ± 170	536 ± 216
Iron intake (mg)	7.9 ± 2.7	7.4 ± 1.8	7.2 ± 2.5
Salt intake (g)	8.7 ± 2.3	8.7 ± 2.9	8.5 ± 2.3
Vitamin A intake (μgRE)	1015 ± 391	1046 ± 512	979 ± 478
Vitamin B1 intake (mg)	0.8 ± 0.4	0.8 ± 0.3	0.9 ± 0.4
Vitamin B2 intake (mg)	1.1 ± 0.3	1.2 ± 0.5	1.1 ± 0.4
Vitamin C intake (mg)	114 ± 66	148 ± 191	118 ± 64
Dietary fiber intake (mg)	14.3 ± 4.3	13.9 ± 4.1	14.0 ± 3.8
Grains (g)	402.5 ± 111.2	449.5 ± 173.4	416.3 ± 133.0
Green and yellow vegetables (g)	121.4 ± 76.1	143.6 ± 170.7	137.8 ± 96.8
Light-colored vegetables (g)	187.1 ± 128.3	165.8 ± 73.0	167.1 ± 78.5
Tubers and roots (g)	65.8 ± 68.1	38.1 ± 50.5	37.2 ± 44.0
Meat (g)	51.9 ± 67.5	67.8 ± 67.2	66.8 ± 73.5
Seafood (g)	97.4 ± 71.4	82.0 ± 56.0	73.3 ± 56.1
Egg (g)	37.4 ± 36.2	40.0 ± 38.9	27.0 ± 26.7
Pulses (g)	61.9 ± 64.1	57.0 ± 59.3	65.6 ± 72.3
Milk and daily product (g)	124.9 ± 119.5	130.1 ± 117.7	142.3 ± 132.1
Fruit (g)	114.5 ± 113.8	109.2 ± 192.1	129.1 ± 128.8
Oil and fat (g)	11.3 ± 10.9	11.9 ± 11.6	9.4 ± 11.6

Statistical differences among the 3 groups were determined by the Kruskal-Wallis and Scheffe's multiple comparison tests. BW: Body weight; RE: Retinol equivalents.

women was significantly lower as liver disease advanced.

Dietary survey

The results of the dietary survey are presented in Table 4. The total nutrition intake and amounts of carbohydrates, protein, and fat were not significantly different among the groups. These results showed that the average food intake of patients was not different compared to that of the controls. Moreover, the intake of important micro-elements such as calcium, iron, salt, vitamins A, B1, B2, and C; and dietary fibers was almost the same in these 3 groups. The food intake and its contents seemed to be sufficient in cirrhotic patients.

Lifestyle survey

The outcomes of the lifestyle survey are described in Table 5. The lifestyle-related questionnaire survey revealed that the amount of alcohol consumed by cirrhotic patients was significantly lower than that of control subjects. Chronic hepatitis patients tended to consume less alcohol compared with control subjects. With regard to drinking habits, significantly more subjects in each patient group replied that they drank less compared to the controls. Significantly more subjects in both patient groups reported that they had enough rest compared to the control group. Furthermore, the sleeping time in patients of the cirrhosis group was significantly longer than that in the other 2 groups. The perceived health status in each patient group was significantly lower than in the control group.

Physical activity survey

Table 6 shows the results of the physical activity survey. The physical activity levels, number of steps, and Ex in the chronic hepatitis and cirrhosis groups were significantly lower than those of the control group. Moreover, TEE in cirrhotic patients was significantly lower than that in control subjects and chronic hepatitis patients.

Results of logistic regression analysis

Table 7 shows the results of univariate and multivariate logistic regression modeling, which were used to determine the relevant factors for insulin resistance. Unadjusted univariate logistic regression modeling suggested that 6 factors, including the presence or absence of hepatocellular carcinoma, AC, Ex, BTR, total energy intake per BW, and percentage fat, were significantly associated with insulin resistance. In the multivariate logistic regression modeling adjusted for sex, age, the presence or absence of hepatocellular carcinoma, AC, Ex, BTR, total energy intake per BW and percentage fat, Ex was a significant factor associated with insulin resistance.

DISCUSSION

In this study, we carried out laboratory evaluations, an-

Table 5 The lifestyle surveys of all subjects

	Control	Chronic hepatitis	Cirrhosis	*P* value
Drinking habit[2]				< 0.001
Nothing	10 (8.8)	40 (67.8)	63 (85.1)	
Sometimes	6 (22.2)	11 (18.6)	9 (12.2)	
Daily	11 (40.7)	8 (13.6)	2 (2.7)	
Alcohol intake (g/d)[1]	13.7 ± 26.6[a]	4.9 ± 14.2	1.6 ± 9.9	
Stage model of alcohol drinking[2]				0.141
Maintenance	4 (23.5)	7 (36.8)	4 (36.4)	
Action	1 (5.9)	1 (5.3)	0 (0)	
Sometimes	3 (17.6)	6 (31.6)	5 (45.5)	
Preparation	0 (0)	1 (5.3)	0 (0)	
Contemplation	9 (52.9)	2 (10.5)	1 (9.1)	
Pre-contemplation	0 (0)	2 (10.5)	1 (9.1)	
Experience of dieting[2]				0.258
No	21 (77.8)	37 (62.7)	59 (79.7)	
Yes	6 (22.2)	22 (37.3)	15 (20.3)	
Stage model of dietary habit[2]				
Maintenance	6 (22.2)	10 (16.9)	12 (16.2)	0.540
Action	2 (7.4)	1 (1.7)	2 (2.7)	
Preparation	2 (7.4)	13 (22)	11 (14.9)	
Contemplation	9 (33.3)	7 (11.9)	19 (25.7)	
Pre-contemplation	8 (29.6)	28 (47.5)	30 (40.5)	
Eating speed (eating lunch within 10 min)[2]				
Fast	8 (29.6)	22 (37.3)	20 (27)	0.451
General or slow	19 (70.4)	37 (62.7)	54 (73)	
Eating and drinking				
Prior to bedtime[2]				
Yes	6 (22.2)	12 (20.3)	16 (21.6)	0.975
No	21 (77.8)	47 (79.7)	58 (78.4)	
Skipping a meal[2]				
Yes	4 (14.8)	18 (30.5)	14 (18.9)	0.700
No	23 (85.2)	41 (69.5)	60 (81.1)	
Resting[2]				
Enough	9 (33.3)	38 (64.4)	49 (66.2)	0.026
Almost enough	9 (33.3)	4 (6.8)	11 (14.9)	
A little shortage	7 (25.9)	14 (23.7)	10 (13.5)	
Shortage	2 (22.2)	3 (5.1)	4 (5.4)	
Sleeping time (h)[1]	6.7 ± 1.1[b]	6.8 ± 1.5[c]	7.6 ± 1.6	
Perceived health status[2]				< 0.001
Very healthy	2 (7.4)	4 (6.8)	3 (4.1)	
Almost healthy	23 (85.2)	32 (54.2)	28 (37.8)	
A little unhealthy	2 (7.4)	18 (30.5)	34 (45.9)	
Unhealthy	0 (0)	5 (8.5)	9 (12.2)	
Stage model of exercise[2]				0.630
Maintenance	12 (44.4)	23 (39)	27 (36.5)	
Action	0 (0)	0 (0)	0 (0)	
Preparation	4 (14.8)	5 (8.5)	13 (17.6)	
Contemplation	10 (37)	23 (39)	25 (33.8)	
Pre-contemplation	1 (3.7)	8 (13.6)	9 (12.2)	
Smoking custom[2]				0.140
Yes	5 (18.5)	13 (22)	8 (10.8)	
No	22 (81.5)	46 (78)	66 (89.2)	
Time to smoking cigarette first after it get out of bed[2]				0.555
Within 5 min	2 (40)	1 (7.1)	1 (14.3)	
From 6 to 30 min	1 (20)	1 (7.1)	1 (14.3)	
From 31 to 60 min	2 (40)	4 (28.6)	4 (57.1)	
More than 61 min	0 (0)	8 (57.1)	1 (14.3)	
Number of smoking cigarettes on a day[2]				0.584
Less than 10 cigarettes	3 (60)	3 (21.4)	2 (28.6)	
From 10 to 20 cigarettes	2 (40)	7 (50)	5 (71.4)	
From 21 to 30 cigarettes	0 (0)	3 (21.4)	0 (0)	
More than 31cigarettes	0 (0)	1 (7.1)	0 (0)	
Period of no smoking[2]				0.356
More than 10 yr	3 (60)	11 (61.1)	16 (72.7)	
From 3 to 10 yr	0 (0)	6 (33.3)	4 (18.2)	
From 1 to 3 yr	1 (20)	0 (0)	1 (4.5)	
From 6 mo to less than 1 yr	1 (20)	1 (5.6)	1 (4.5)	

Stage model of smoking[2]				0.481
Maintenance	5 (50)	19 (59.4)	22 (71)	
Action	0 (0)	0 (0)	0 (0)	
Preparation	0 (0)	0 (0)	3 (9.7)	
Contemplation	4 (40)	7 (21.9)	3 (9.7)	
Pre-contemplation	1 (10)	6 (18.8)		

[a]$P = 0.003$, [b]$P = 0.028$, [c]$P = 0.007$ *vs* liver cirrhosis. Data are expressed as mean ± SD or as number of patients. [1,2]Statistical differences among the 3 groups were determined by the Kruskal-Wallis and Scheffe's multiple comparison tests[1] or Spearman's rank correlation coefficient[2].

Table 6 The physical activity surveys of the subjects (mean ± SD)

	Control	Chronic hepatitis	Cirrhosis
n	27	43	53
TEE (kcal/d)	1781 ± 272[d]	1732 ± 305[h]	1593 ± 220
Physical activity (kcal/d)	210 ± 113[a,e]	151 ± 95	125 ± 74
Number of steps (step/d)	8070 ± 3027[b,f]	6011 ± 2952	5789 ± 3368
Ex (Ex/wk)	12.4 ± 9.3[c,g]	7.6 ± 6.3	7.0 ± 7.7

[a]$P = 0.035$, [b]$P = 0.032$, [c]$P = 0.038$ *vs* chronic hepatitis; [d]$P = 0.013$, [e]$P = 0.001$, [f]$P = 0.011$, [g]$P = 0.013$, [h]$P = 0.042$ *vs* liver cirrhosis. Statistical differences among the 3 groups were determined by the Kruskal-Wallis and Scheffe's multiple comparison tests. TEE: Total energy expenditure; Ex: Exercise.

thropometric assessments, a diet, lifestyle, and physical activity survey in chronic liver disease patients. The results were compared with the data of healthy subjects. As shown in Table 2, a considerable number of the chronic hepatitis patients presented insulin resistance without obvious elevation of FBS. Thirty-three point nine percent in the chronic hepatitis and 37.8% in cirrhotic patients indicated insulin resistance. Insulin resistance has been reported to be correlated with liver fibrosis[20,21], carcinogenesis[22] and mortality[23] of liver cancer in chronic liver disease patients. FBS as well as IRI should be measured and HOMA-IR should be calculated for an early diagnosis and for improving insulin resistance by diet and Ex therapy.

The rate of obesity in men and women was 30.5% and 20.8%, respectively, according to the national health and nutrition examination survey conducted by the Japanese Ministry of Health, Labour and Welfare in 2009. In this study, the incidence of obesity in men with liver cirrhosis was almost the same as that in the general adult male population. However, the rate of obesity in women was significantly lower as liver disease advanced. However, BMI was not significantly different among the 3 groups, and 72.0% of the women with liver cirrhosis had a normal BMI. These results suggested that almost all women with liver cirrhosis might have normal weight. Complications such as metabolic syndrome, obesity, and fatty liver in patients with chronic liver disease were risk factors of liver fibrillization[24] and carcinogenesis[25]. These complications are related to increasing mortality of carcinoma[11] and decreased response to antiviral therapy[26]. If the condition of patients with obesity or fatty liver could be improved by correcting their diet and lifestyle, these approaches might be effective to curb the progression of the disease, carcinogenesis, and to improve the efficacy of interferon therapy. However, malnutrition in cirrhotic patients is caused by the decreased protein synthesis and disturbed energy metabolism that result from liver failure. Protein malnutrition can cause a decrease in skeletal muscle mass in cirrhotic patients. In fact, AMC and AMA in patients with chronic hepatitis and liver cirrhosis were significantly lower than those in the control subjects, while AC was not significantly different among all groups. Moreover, TSF in the chronic hepatitis group was significantly higher than that in the control group. These results suggest the possibility of a decrease in skeletal muscle mass and a relative increase in fat mass in patients with chronic liver disease. However, anthropometry might underestimate the prevalence and severity of malnutrition in patients with cirrhosis. In particular, the underestimation was more pronounced in patients with Child-Pugh A and B cirrhosis, although anthropometry is a simple and non-invasive method[27]. More attention needs to be paid to the method of nutrition assessment. In the future, the body composition of cirrhotic patients might have to be evaluated again by using more accurate methods such as dual-energy X-ray absorptiometry.

In this study, the handgrip strength in the patient groups was significantly lower than that in the control group. Handgrip strength has been reported to be strongly correlated with muscle mass[28]. Decreased handgrip strength corresponded with lower %AMC and %AMA values. These results might reflect a decrease in muscle mass. Furthermore, the handgrip strength can be used to predict morbidity of complications in cirrhotic patients[29]. Therefore, it may be a valuable predictive factor of malnutrition and its accompanying complications. Although not specific to patients with liver disease, a Japanese study published in 2007 reported that the multivariate-adjusted reactive risk of all causes of death, excluding external causes, for each 5-kg increment of grip strength was significantly lower in middle-aged and elderly individuals[30]. Maintaining grip strength might benefit cirrhotic patients.

In the dietary survey, there were no significant differences in nutrition intake between the groups. Furthermore, patients did not show a deficiency in total energy and protein intake per BW, as per the guidelines of ESPEN Nutritional Management. This finding suggests that dietary intake was almost sufficient to fulfill the

Table 7 Logistic regression model of insulin resistance in patients with chronic liver disease

	Control (*n*)	Case (*n*)	Univariate		Multivariate	
			Odds ratio (95% CI)	*P* value	Odds ratio[1] (95% CI)	*P* value
Viral infection						
HBV infection	15	3	1	0.055		
HCV infection	38	28	3.684 (0.972-13.960)			
Groups						
Chronic hepatitis	25	14	1	0.859		
Liver cirrhosis	28	17	1.084 (0.445-2.639)			
The hepatocellular carcinoma						
Absent	44	19	1	0.030	1	0.290
Present	9	12	3.087 (1.115-8.544)		0.311 (0.036-2.713)	
Sex						
Female	35	18	1	0.466	1	0.700
Male	18	13	1.404 (0.564-3.496)		0.712 (0.126-4.010)	
Age (yr)						
< 65	23	14	1	0.875	1	0.366
≥ 65	30	17	0.931 (0.382-2.271)		0.486 (0.102-2.324)	
%AC						
≥ 100	33	26	1	0.042	1	0.091
< 100	20	5	0.317 (0.105-0.960)		0.223 (0.039-1.271)	
%TSF						
≥ 100%	31	24	1	0.083		
< 100%	22	7	0.411 (0.151-1.121)			
%AMC						
≥ 100%	35	22	1	0.641		
< 100%	18	9	0.795 (0.304-2.081)			
%AMA						
≥ 100%	38	22	1	0.943		
< 100%	15	9	1.036 (0.389-2.759)			
Handgrip strength (%)						
≥ 69.0	22	17	1	0.360		
< 69.0	30	13	1.526 (0.617-3.771)			
Ex (Ex/wk)						
≥ 4.0	29	7	1	0.003	1	0.024
< 4.0	10	14	5.800 (1.823-18.455)		6.809 (1.288-36.001)	
Plt ($\times 10^2/\mu L$)						
≥ 10.0	37	15	1	0.054		
< 10.0	16	16	2.466 (0.986-6.168)			
Alb (g/dL)						
≥ 3.5	43	20	1	0.094		
< 3.5	10	11	2.365 (0.864-6.476)			
BTR						
≥ 4.41	31	11	1	0.025	1	0.238
< 4.41	17	18	2.984 (1.148-7.756)		2.770 (0.509-15.061)	
Energy intake per BW (kcal/BW)						
≤ 30	21	22	1	0.007	1	0.602
> 30	32	9	0.268 (0.104-0.695)		0.689 (0.171-2.785)	
Protein intake per BW (g/BW)						
≤ 1.2	24	17	1	0.339		
> 1.2	29	14	0.682 (0.280-1.660)			
Fat percentage (%)						
≤ 25	26	23	1	0.027	1	0.096
> 25	27	8	0.335 (0.127-0.882)		0.222 (0.038-1.307)	

[1]Adjusted for the presence or absence of hepatocellular carcinoma, arm circumference (AC), exercise (Ex), molar ratio of branched-chain amino acid to tyrosine (BTR), total energy intake per body weight (BW), and percentage fat, age. HBV: Hepatitis B virus; HCV: Hepatitis C virus; TSF: Triceps skin fold thickness; AMC: Arm muscle circumference; AMA: Arm muscle area; Plt: Platelet count; Alb: Albumin.

nutritional requirements. The results of the biochemical blood tests and anthropometric assessments showed that cirrhotic patients were malnourished, leading to reduced values of serum albumin, AMC, AMA and handgrip strength. Although protein intake was adequate in the cirrhosis group, the BTR was low. This result suggests that improving the amino acid imbalance by dietetic treatment is difficult. Therefore, oral BCAA supplementation in cirrhotic patients has been shown to be effective in improving BTR and maintaining serum albumin and their quality of life (QoL)[31]. In addition, oral BCAA supplementation could decrease the risk of specific hepatic carcinogenesis in cirrhotic patients[10].

In the lifestyle survey, chronic liver disease patients

consumed significantly less alcohol and had a lower drinking frequency. These results suggested that the patients were aware of the adverse effect of alcohol drinking. Cirrhotic patients had a significantly longer sleeping time, and more patients reported that their resting time was sufficient than the other 2 groups. This shows that patients with cirrhosis are careful in maintaining rest. Perceived health status in the patient groups was significantly lower than that in the control group; progression of the liver disease might influence the QoL of the patients.

The result of the physical activity survey suggested that cirrhotic patients in urban areas had significantly lower TEE than the control and chronic hepatitis groups. In a study that determined REE by using an indirect calorimeter, the REE of cirrhotic patients was found to vary[32]. However, the REE in cirrhotic patients was significantly higher than that in the control and chronic hepatitis groups when measured using a direct calorimeter[33]. The Lifecorder of TEE was conjectured by body motion, but this measured value does not consider the increased REE in cirrhotic patients. This suggested that the TEE of cirrhotic patients in this study might be underestimated. However, a study that validated the Lifecorder reported that there was a strong correlation between physical activity levels and measured METs while walking[17]. At the very least, this result suggested that the level of physical activity of chronic hepatitis and cirrhosis group was low. As mentioned above, the perceived health status in cirrhotic patients was also low. We think that all physical activity might be suppressed by stressing about the need for rest in the usual life guidelines for cirrhosis. Some reports stated that maintenance of weight loss and Ex in overweight patients with liver disease resulted in a sustained improvement in liver function, insulin resistance and QoL[34,35]. Obese cirrhotic patients would rather try to increase the intensity of daily activities involving Ex than continue to maintain excessive resting. However, since patients with decompensated cirrhosis with ascites, hepatic encephalopathy, and jaundice need to lie quietly and consume sufficient amounts of nutrients, we should probably exclude them from the patients with chronic hepatitis and compensated cirrhosis enrolled in this study.

In this study, univariate and multivariate logistic regression modeling were used to identify the relevant factors for insulin resistance. The results show that decreased Ex was a significant factor for insulin resistance. For patients with chronic liver disease, increasing the amount of physical activity might improve insulin resistance. Insulin resistance tended to decrease at low values of %AC, which is an index of muscle and fat mass. In this study, the muscle mass in patients with chronic liver disease was lower than in the control group. While the amount of fat mass in the liver cirrhosis group was not significantly different from that of the control group, the amount of fat mass in the chronic hepatitis group was higher than that of the control group. In patients with chronic liver disease, obesity or muscle wasting might be related to insulin resistance. Insulin resistance tended to decrease at higher fat percentages. Insulin secretion might not be as easily stimulated when fat is ingested compared with when carbohydrates are ingested. This might be associated with the insulin resistance observed in chronic liver disease. However, this study has limitations. We have not completely verified a causal relationship between Ex and insulin resistance as this is a cross sectional study. It will thus be necessary to perform a future cohort study to verify whether insulin resistance in patients with cirrhosis is directly induced by decreased physical activity.

In summary, the results of this study suggested that chronic liver disease patients in urban areas have lower skeletal muscle mass and higher relative body fat percentage. We think that the lower skeletal muscle mass may result from the decline in BTR, which indicates an amino acid imbalance, and that the intensity of daily activities was lower because of excessive resting instead of a decrease in dietary intake. Moreover, this study showed that insulin resistance might exist in early-stage chronic liver disease patients. Ex was a relevant factor for insulin resistance, and decreased physical activity was associated with insulin resistance.

Therefore, non-hospitalized cirrhotic patients in urban areas need to maintain an adequate dietary intake and receive lifestyle guidance to increase their physical activity levels.

COMMENTS

Background

Cirrhosis is an end-stage chronic liver disease, and one of the typical diseases that is associated with malnutrition. However, the combination of over-nutrition and obesity in cirrhosis patients indicates the possibility of an impaired pathogenesis. These findings suggest that excessive food intake, decreased intensity of daily activity, and a lack of exercise (Ex) in cirrhotic patients promotes obesity, fatty liver, and hepatic carcinogenesis. Few researchers have studied the effect of dietary habits and lifestyle in non-hospitalized cirrhotic patients on their conditions.

Research frontiers

Recently, both experimental and clinical studies found that over-nutrition, fatty liver, and glucose intolerance are significant risk factors of hepatic carcinogenesis. Moreover, insulin resistance has been reported to be correlated with liver fibrosis and mortality of liver cancer in chronic liver disease patients. Some reports have said that maintenance of weight loss and Ex in overweight patients with liver disease resulted in a sustained improvement in liver function, insulin resistance, and quality of life. To improve insulin resistance or the loss of excess weight, correction of malnutrition and the addition of moderate Ex are effective methods of therapy. However, it is not clear if Ex that is moderate in intensity or quantity will be effective therapy for cirrhotic patients. The purpose of this study is to assess the nourishment status of non-hospitalized patients with compensated cirrhosis.

Innovations and breakthroughs

The results of this study suggested that chronic liver disease patients in urban area have lower skeletal muscle mass and higher relative body fat percentage. Moreover, this study showed that insulin resistance might exist in early-stage chronic liver disease patients. Ex was a relevant factor for insulin resistance, and decreased physical activity was associated with insulin resistance.

Applications

The results of this study suggest that non-hospitalized cirrhotic patients in urban areas need to maintain an adequate dietary intake and receive lifestyle guidance to increase their physical activity levels. Decreased physical activity was

associated with insulin resistance. Therefore, increasing physical activity may improve insulin resistance and suppress hepatocellular carcinogenesis.

Terminology

Ex is a unit to express the quantity of physical activity, calculated by multiplying metabolic equivalents (METs) by the duration of the activity (h) as proposed in the Physical activity guide for Health Promotion 2006, which was published by the Ministry of Health, Labor and Welfare of Japan. METs is an index for the intensity of physical activity, calculated as energy expenditure (oxygen uptake, mL/kg per minute) during a specific physical activity divided by sitting/resting energy expenditure.

Peer review

In this paper, the authors investigated the nourishment status and lifestyle of non-hospitalized patients with compensated cirrhosis by using non-invasive methods in Japan. They found that insulin resistance was seen in more than 35% of patients with chronic liver disease. In addition, the handgrip strength, physical activity levels, number of steps and Ex were significantly lower in the cirrhotic group than in the control group. The authors concluded that non-hospitalized cirrhotic patients may need to maintain an adequate dietary intake and receive lifestyle guidance in order to increase their physical activity levels. Overall, theseindings are interesting.

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BRIEF ARTICLE

Correlations between endoscopic and clinical disease activity indices in intestinal Behcet's disease

Hyun Jung Lee, Youn Nam Kim, Hui Won Jang, Han Ho Jeon, Eun Suk Jung, Soo Jung Park, Sung Pil Hong, Tae Il Kim, Won Ho Kim, Chung Mo Nam, Jae Hee Cheon

Hyun Jung Lee, Hui Won Jang, Han Ho Jeon, Eun Suk Jung, Soo Jung Park, Sung Pil Hong, Tae Il Kim, Won Ho Kim, Jae Hee Cheon, Department of Internal Medicine, Institute of Gastroenterology, Yonsei University College of Medicine, Seoul 120-752, South Korea
Youn Nam Kim, Department of Biostatistics, Yonsei University College of Medicine, Seoul 120-752, South Korea
Chung Mo Nam, Department of Preventive Medicine, Yonsei University College of Medicine, Seoul 120-752, South Korea
Author contributions: Lee HJ performed the data analysis and wrote the manuscript; Cheon JH had original idea on this subject, performed colonoscopy, performed the data analysis and wrote the manuscript; Jang HW, Jeon HH, Jung ES, Park SJ, Hong SP, Kim TI and Kim WH performed colonoscopy and reviewed the manuscript critically; Kim YN and Nam CM performed the statistical analysis and reviewed the manuscript.
Correspondence to: Jae Hee Cheon, MD, PhD, Division of Gastroenterology, Department of Internal Medicine, Institute of Gastroenterology, Yonsei University College of Medicine, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-752, South Korea. geniushee@yuhs.ac
Telephone: +82-2-22281990 Fax: +82-2-3936884
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Abstract

AIM: To develop a novel endoscopic severity model of intestinal Behcet's disease (BD) and to evaluate its feasibility by comparing it with the actual disease activity index for intestinal Behcet's disease (DAIBD).

METHODS: We reviewed the medical records of 167 intestinal BD patients between March 1986 and April 2011. We also investigated the endoscopic parameters including ulcer locations, distribution, number, depth, shape, size and margin to identify independent factors associated with DAIBD. An endoscopic severity model was developed using significant colonoscopic variables identified by multivariate regression analysis and its correlation with the DAIBD was evaluated. To determine factors related to the discrepancy between endoscopic severity and clinical activity, clinical characteristics and laboratory markers of the patients were analyzed.

RESULTS: A multivariate regression analysis revealed that the number of intestinal ulcers ($\geqslant$ 2, P = 0.031) and volcanoshaped ulcers (P = 0.001) were predictive factors for the DAIBD. An endoscopic severity model (Y) was developed based on selected endoscopic variables as follows: Y = 47.44 + 9.04 × non-Ileocecal area + 11.85 × $\geqslant$ 2 of intestinal ulcers + 5.03 × shallow ulcers + 12.76 × deep ulcers + 4.47 × geographic-shaped ulcers + 26.93 × volcano-shaped ulcers + 8.65 × $\geqslant$ 20 mm of intestinal ulcers. However, endoscopic parameters used in the multivariate analysis explained only 18.9% of the DAIBD variance. Patients with severe DAIBD scores but with moderately predicted disease activity by the endoscopic severity model had more symptoms of irritable bowel syndrome (21.4% *vs* 4.9%, P = 0.026) and a lower rate of corticosteroid use (50.0% *vs* 75.6%, P = 0.016) than those with severe DAIBD scores and accurately predicted disease by the model.

CONCLUSION: Our study showed that the number of intestinal ulcers and volcano-shaped ulcers were predictive factors for severe DAIBD scores. However, the correlation between endoscopic severity and DAIBD (r = 0.434) was weak.

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Key words: Intestinal Behcet's disease; Disease activity index; Colonoscopy; Ulcer; Endoscopic severity

Peer reviewers: Yuji Naito, Professor, Department of Medical Proteomics, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan; Francis Seow-Choen,

Professor, Seow-Choen Colorectal Centre, Mt Elizabeth Hospital, 290 Orchard Road Paragon Shopping Centre No. 06-06, Singapore 238859, Singapore

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INTRODUCTION

Behcet's disease (BD) is a chronic, relapsing, multi-systemic inflammatory disorder characterized by recurrent oral and genital ulcers combined with other systemic manifestations. The systemic manifestations are due to involvement of many other organs including the skin, joints, blood vessels, nervous system, epididymis, and gastrointestinal tract[1,2]. In particular, when patients with BD have predominant gastrointestinal symptoms and show intestinal ulcerations according to objective measures, the disease is then diagnosed as "intestinal BD"[3,4].

Intestinal BD is generally accepted as a type of inflammatory bowel disease (IBD), a group of diseases that includes ulcerative colitis (UC) and Crohn's disease (CD). Because IBD has various, fluctuated clinical courses with repeated remission and relapse, the disease activity index is helpful for disease management and therefore has been commonly used in monitoring treatment response. For example, disease activity is usually defined according to the Truelove and Witts' criteria[5] or the Mayo score system[6] as mild, moderate, or severe disease for UC and measured by the Crohn's disease activity index (CDAI)[7] or the Harvey-Bradshaw index[8] for CD.

Endoscopy is by far the best and most widely used modality for assessment of the disease extent and severity of IBD[9]. A study investigating a cohort of CD patients under long-term treatment with infliximab recently reported that mucosal healing was associated with a significantly lower need for abdominal surgery[10]. Furthermore, data from the Active Ulcerative Colitis Trials 1 and 2[11] have suggested that mucosal healing was associated with a better outcome in UC, more specifically, a decreased risk of relapse. Therefore, the macroscopic mucosal appearance is considered a valuable indicator of disease activity. In this regard, colonoscopic activity has been assessed using a modified version of Baron's criteria[12] for UC and the Crohn's disease endoscopic index of severity (CDEIS)[13] for CD.

Although the disease activity index for intestinal Behcet's disease (DAIBD), a relatively simple eight-point system, was recently introduced[14], there are no specific tools for the measurement of endoscopic severity in intestinal BD. There have been several descriptions of clinical outcomes according to colonoscopic findings[15,16], however, endoscopic appearance has never been taken into account in evaluating disease activity in intestinal BD. Therefore, the aim of this study was to identify endoscopic factors associated with the DAIBD, to develop a novel endoscopic severity model of intestinal BD using these factors, and to evaluate the correlations between the actual DAIBD and endoscopic severity.

MATERIALS AND METHODS

Patients

We reviewed the medical records of patients with intestinal BD at Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea, between March 1986 and April 2011. Patients with a history of previous colon operations were excluded from the study and those diagnosed or suspected as having UC, CD, intestinal tuberculosis, infectious colitis, or malignancy were not included in our study.

BD was diagnosed according to the criteria from the 1987 Behcet's Disease Research Committee of Japan[17] and was divided into the following three types: complete, incomplete and suspected BD. Intestinal involvement of BD was evaluated using colonoscopy for the large bowel and terminal ileum involvement and small bowel follow-through or computed tomography enterography for the small bowel involvement in patients with BD who had gastrointestinal symptoms. Diagnosis of intestinal BD was made according to the criteria as previously described using a modified Delphi process[2,18]. Patients were categorized into three groups as definite, probable, and suspected on the basis of colonoscopic findings and extraintestinal systemic manifestations. Clinical activity of intestinal BD was judged by the DAIBD composed of the following eight variables: general well-being, fever, extraintestinal manifestations, abdominal pain, abdominal mass, intestinal complications, and number of liquid stools in one week. The DAIBD was classified into quiescent ($\leqslant$ 19), mild (20-39), moderate (40-74), and severe disease ($\geqslant$ 75)[14].

Methods

We collected demographic data including initial gastrointestinal symptoms and other extraintestinal manifestations related to systemic BD at the time of colonoscopy. We also investigated all colonoscopic findings with respect to location, distribution, number, depth, shape, size, and ulcer margin. Patients with < 5 ulcers that were oval in shape, deep, with discrete borders, and located in the ileocecal area including the terminal ileum, ileocecal valve, and cecum were considered as having typical intestinal BD lesions[19]. When the colon was divided into five segments (ileocecal area, ascending colon, transverse colon, descending colon, and rectosigmoid colon), a localized type was defined when lesions were restricted to only one segment and a diffuse type was defined when lesions were scattered in more than two colonic segments[15]. Furthermore, when two or more ulcers were observed, the largest diameter of the biggest ulcer was

used for the measurement of depth, shape, size and margin for analysis. This study was approved by the Institutional Review Board of Yonsei University College of Medicine and was conducted in accordance with the ethical principles of the Declaration of Helsinki.

Statistical analysis

Continuous variables are presented as mean ± SD and categorical variables as frequency and percentage. An endoscopic severity model was constructed using the DAIBD as a dependent variable and the following endoscopic parameters as explanatory variables: ulcer location, distribution, number, depth, shape, size, and margin. To identify independent factors associated with disease activity for intestinal BD, we selected explanatory items using univariate analysis ($P < 0.05$) and also items that were recommended to be included by experts, which were followed by multiple linear regression analysis. The independent variables were evaluated using Student's *t*-test or one-way analysis of variance, with post hoc tests for confirmation of significance (Tukey's test at $\alpha = 0.05$). R^2 in the regression is used for how much the variance of the DAIBD can be explained by the endoscopic parameters. Moreover, relationships between predicted scores and the DAIBD were evaluated using Spearman's correlation coefficients.

Using an endoscopic severity model, we sought to predict actual disease activity for intestinal BD. To evaluate factors associated with any discrepancy between the predicted score using an endoscopy severity model and the actual DAIBD, we divided patients into four groups (quiescent, mild, moderate, and severe groups) according to their actual DAIBD cutoff scores and analyzed clinical characteristics and laboratory markers in each group. They were further classified according to their predicted DAIBD scores using the endoscopic model. Two of the groups of patients with the actual DAIBD scores showed values consistent with the predicted scores by the endoscopic model, while the other two groups of patients showed disagreement between the actual DAIBD and predicted scores; one with lower predicted scores than the actual DAIBD scores and the other with higher predicted scores than the actual DAIBD scores. Student's *t*-test was used to compare continuous variables and chi-squared test was used to compare categorical variables for each group.

$P < 0.05$ was considered statistically significant for all tests. All statistical analyses were performed using SPSS Version 17.0 for Windows (SPSS Inc., Chicago, IL, United States) and/or SAS Version 9.1 (SAS Institute, Gary, NC, United States).

RESULTS

Baseline clinical and colonoscopic characteristics of patients with intestinal BD

A total of 243 patients with intestinal BD were recruited during the study period, and 72 of 243 patients were excluded because they underwent operations such as ileocecectomy, right hemicolectomy, or small bowel resection before undergoing colonoscopy. One patient was excluded after finally being diagnosed with CD after follow-up colonoscopy, and 3 patients were further excluded who were confirmed not to have intestinal BD at the end of the follow-up period. The remaining 167 patients were included in this study. Their clinical and colonoscopic characteristics are summarized in Tables 1 and 2, respectively.

The mean age at diagnosis was 38.5 years (range:15-73 years) for BD and 41.4 years (range: 16-73 years) for intestinal BD. Ninety-six patients (57.5%) were female. According to the diagnostic criteria for BD, 3 patients (1.8%) had the complete type, 78 (46.7%) had the incomplete type, and 53 (31.7%) had the suspected type of disease. Twenty-five patients (15.0%) presented with only typical oral ulcers and 8 (4.8%) had only gastrointestinal lesions without any extraintestinal manifestations. The chief complaints of patients were diarrhea (85.0%), abdominal pain (71.8%), and abdominal tenderness (71.2%). At the time of colonoscopy, 5-aminosalicylic acids or sulfasalazine were prescribed to almost all the patients with intestinal BD (97.6%), azathioprine or 6-mercaptopurine to 24 patients (14.4%), colchicine to 80 (47.9%), and corticosteroids to 72 (43.1%).

On colonoscopy, the ileocecal area was the most common location for intestinal lesions (156 patients, 93.4%), with ileal ulcers present in 4 (2.4%). Localized intestinal involvement was found in 142 patients (85.0%) and 86 (51.5%) had a solitary intestinal ulcer. Regarding the depth and shape of intestinal ulcers, 109 patients (65.3%) had deep ulcers and 79 (47.3%) had oval-shaped ulcers. Patients with intestinal BD had relatively large ulcers; ulcers larger than 20 mm were noted in 82 patients (49.1%). The ulcer margins were usually discrete (75 patients, 44.9%), and 94 patients (56.3%) had typical ulcers. With regard to the intestinal BD diagnostic criteria, 69 (41.3%), 82 (49.1%), and 16 patients (9.6%) were classified as definite, probable, and suspected types, respectively.

Endoscopic factors associated with disease activity for intestinal BD

Univariate analysis revealed that the depth of intestinal ulcers ($F = 5.03$, $P = 0.008$), ulcer shape ($F = 13.33$, $P < 0.001$), and ulcer size ($F = 6.62$, $P < 0.001$) were significantly associated with disease activity for intestinal BD (Table 3). The mean DAIBD score of deep ulcers was significantly greater than that of shallow ulcers as shown by Tukey's multiple comparison test ($P = 0.027$) and the mean DAIBD score of volcano-shaped ulcers was also greater than that of oval-shaped or geographic ulcers ($P < 0.001$ and $P = 0.001$, respectively). The mean DAIBD score of ulcers larger than 20 mm was significantly greater than that of ulcers less than 5 mm or ulcers ranging from 10 mm to 20 mm ($P = 0.014$ and $P = 0.002$, respectively). No difference in the DAIBD

Table 1 Baseline clinical characteristics of patients with intestinal Behcet's disease

Characteristic	No. of patient (%)
Age at diagnosis of BD (yr)	38.5 ± 12.2
Age at diagnosis of intestinal BD (yr)	41.4 ± 12.3
Gender (male:female)	71:96 (42.5:57.5)
Systemic symptoms and signs of BD	
Recurrent oral ulcer	159 (95.2)
Recurrent genital ulcer	78 (46.7)
Ocular lesion	10 (6.0)
Skin lesion	82 (49.1)
Positive pathergy test	4 (2.4)
Arthritis or arthralgia	66 (39.5)
Neurologic lesion	0
Vascular lesion	2 (1.2)
Clinical subtype of BD	
Complete	3 (1.8)
Incomplete	78 (46.7)
Suspected	53 (31.7)
Symptoms and signs of intestinal involvement	
Abdominal pain	120 (71.8)
Diarrhea	142 (85.0)
Abdominal mass	4 (2.4)
Tenderness	119 (71.2)
Nausea/vomiting	4 (2.4)
Melena/hematochezia	31 (18.6)
Fistula	4 (2.4)
Stenosis (intestinal obstruction)	10 (6.0)
Abscess	0
Perforation	0
Current use of medication	
5-ASA or sulfasalazine	163 (97.6)
Azathioprine or 6-mercaptopurine	24 (14.4)
Colchicine	80 (47.9)
Corticosteroid	72 (43.1)
Patient global assessment	
Well	63 (37.7)
Mild	53 (31.7)
Moderate	45 (26.9)
Severe	6 (3.6)
DAIBD	74.9 ± 36.1 (5-155)
Quiescent (≤ 19)	2 (1.2)
Mild (20-39)	32 (19.2)
Moderate (40-74)	50 (29.9)
Severe (≥ 75)	83 (49.7)

BD: Behcet's disease; DAIBD: Disease activity index for intestinal Behcet's disease; 5-ASA: 5-aminosalicylic acid.

Table 2 Baseline colonoscopic characteristics of patients with intestinal Behcet's disease

Characteristic	No. of patient (%)
Location of intestinal lesion	4 (2.4)
Ileal area	156 (93.4)
Ileocecal area	25 (15.0)
Ascending colon	18 (10.8)
Other colonic segment	
Distribution of intestinal lesion	
Localized involvement	142 (85.0)
Diffuse involvement	25 (15.0)
Number of intestinal ulcer	
Solitary	86 (51.5)
2-5	53 (31.7)
≥ 5	28 (16.8)
Depth of intestinal ulcer	
Aphthous	12 (7.2)
Shallow	46 (27.5)
Deep	109 (65.3)
Shape of intestinal ulcer	
Oval	79 (47.3)
Geographic	52 (31.1)
Volcano	36 (21.6)
Size of intestinal ulcer (mm)	
< 5	11 (6.6)
5-10	29 (17.4)
10-20	45 (26.9)
≥ 20	82 (49.1)
Margin of intestinal ulcer	
Discrete	75 (44.9)
Elevation	13 (7.8)
Nodular elevation	32 (19.2)
Marginal erythema	47 (28.1)
Type of intestinal ulcer	
Typical type	94 (56.3)
Atypical type	73 (43.7)
Diagnostic criteria of intestinal BD	
Definite	69 (41.3)
Probable	82 (49.1)
Suspected	16 (9.6)

BD: Behcet's disease.

score was observed with regard to any other endoscopic variables including location, distribution, number, or pattern of ulcer margin.

Significant variables ($P < 0.05$) found on univariate analysis were included in the multivariate analysis to identify independent factors associated with disease activity. Location and number of ulcers that experts have recommended as potentially important predictive factors were also included in the multivariate analysis. Multivariate regression analysis showed that the number of intestinal ulcers (≥ 2, $P = 0.031$) and that of volcano-shaped ulcers ($P = 0.001$) were independent predictive factors for the DAIBD (Table 4).

Using the coefficients from the estimated regression model (Table 4), we generated an endoscopic severity model to predict DAIBD scores (Y) based on five optimal selected endoscopic variables. The final model was as follows: Y = 47.44 + 9.04 × non-ileocecal area + 11.85 × ≥ 2 of intestinal ulcers + 5.03 × shallow ulcers + 12.76 × deep ulcers + 4.47 × geographic-shaped ulcers + 26.93 × volcano-shaped ulcers + 8.65 × ≥ 20 mm of intestinal ulcers.

Predictive factors for discrepancies between predicted endoscopic score and actual DAIBD

However, the five most predictive endoscopic parameters in the multiple linear regression analysis explained only 18.9% of the DAIBD variance. Therefore, we attempted to find out the reasons for this unexpected discrepancy. Using the endoscopic severity model, the predicted DAIBD severity distribution was as follows: 108 patients (64.7%) had moderate disease activity and 59 (35.3%) had severe disease activity (Table 5). Conversely, in this study, the actual DAIBD scores demonstrated that disease activity was quiescent in 2 patients (1.2%), mild in 32 (19.2%), moderate in 50 (29.9%), and severe in 83 (49.7%).

Table 3 Univariate analysis of endoscopic factors associated with the disease activity index for intestinal Behcet's disease

Endoscopic factor	DAIBD (mean ± SD)	*F*/*t* value	*P* value
Location		0.48	0.632
Ileocecal area	74.58 ± 36.74		
Other	80.00 ± 26.08		
Distribution			
Localized	73.98 ± 35.22	-0.82	0.414
Diffuse	80.40 ± 41.10		
Number of intestinal ulcer		0.39	0.679
Solitary	72.67 ± 30.92		
2- 5	76.51 ± 41.75		
≥ 5	78.93 ± 40.08		
Depth of intestinal ulcer		5.03	0.008
Aphthous	57.08 ± 37.02		
Shallow	65.00 ± 30.09		
Deep	81.10 ± 37.01		
Shape of intestinal ulcer		13.33	< 0.001
Oval	65.13 ± 37.79		
Geographic	72.60 ± 28.40		
Volcano	99.86 ± 30.95		
Size of intestinal ulcer (mm)		6.62	< 0.001
< 5	52.73 ± 35.45		
5-10	69.48 ± 36.38		
10-20	63.00 ± 33.02		
≥ 20	86.40 ± 34.29		
Margin of intestinal ulcer		1.95	0.124
Discrete	80.47 ± 37.70		
Elevation	56.54 ± 33.63		
Nodular elevation	75.47 ± 29.93		
Marginal erythema	70.85 ± 36.81		

DAIBD: Disease activity index for intestinal Behcet's disease.

Table 4 Multivariate analysis of endoscopic factors associated with the disease activity index for intestinal Behcet's disease

Endoscopic factor	β coefficient	*P* value
Intercept	47.44	
Location		
Ileocecal area	0	
Other	9.04	0.389
Number of intestinal ulcer		
Solitary	0	
≥ 2	11.85	0.031
Depth of intestinal ulcer		
Aphthous	0	
Shallow	5.03	0.651
Deep	12.76	0.261
Shape of intestinal ulcer		
Oval	0	
Geographic	4.47	0.495
Volcano	26.93	0.001
Size of intestinal ulcer (mm)		
< 20	0	
≥ 20	8.65	0.225

Table 5 Predicted and actual disease activity index for intestinal Behcet's disease scores in intestinal Behcet's disease patients

Actual DAIBD	Predicted DAIBD by an endoscopic model				Total
	Quiescent (≤19)	Mild (20-39)	Moderate (40-74)	Severe (≥ 75)	
Quiescent (≤ 19)	0	0	1	1	2
Mild (20-39)	0	0	28	4	32
Moderate (40-74)	0	0	37	13	50
Severe (≥ 75)	0	0	42	41	83
Total	0	0	108	59	167

DAIBD: Disease activity index for intestinal Behcet's disease.

To find out which predictive factors are causing discrepancies between the predicted endoscopic scores and the actual DAIBD scores, we first analyzed 83 patients with severe actual DAIBD scores. Among the 83 patients, 42 (50.6%) had moderate and 41 (49.4%) had severe predicted disease activity based on the endoscopic severity model. Moderately predicted group had more irritable bowel syndrome symptoms (21.4% *vs* 4.9%, $P = 0.026$) and a lower rate of corticosteroid use (50.0% *vs* 75.6%, $P = 0.016$) than severely predicted group. However, infection rates (16.7% *vs* 12.2%, $P = 0.562$) and rates of systemic BD presentation (57.1% *vs* 46.3%, $P = 0.325$) were not significantly different between the two groups.

Among the remaining 84 patients with mild or moderate DAIBD, 37 patients (44.0%) showed consistent results between the actual DAIBD and their predicted scores, while 47 (56.0%) showed inconsistent results. Clinical or laboratory variables including rate of corticosteroid use (25.5% *vs* 21.6%, $P = 0.676$), erythrocyte sedimentation rate (ESR) (39.15 ± 27.66 mm/h *vs* 33.74 ± 27.98 mm/h, $P = 0.421$), or C-reactive protein (CRP) level (7.11 ± 18.33 mg/L *vs* 2.81 ± 3.99 mg/L, $P = 0.162$) were not significantly different between the two groups.

DISCUSSION

To the best of our knowledge, there have been no studies concerning endoscopic factors associated with disease activity for intestinal BD. Moreover, there is currently no available endoscopic severity model for intestinal BD. To date, the management of intestinal BD and the assessment of its response to treatment depend heavily on expert opinions. Although recently, the DAIBD was proposed to objectively evaluate disease activity, which would be useful to physicians in clinical practice[14], endoscopy has rarely been investigated as a predictor of disease activity in intestinal BD. Accordingly, we aimed to identify endoscopic factors related to disease activity of intestinal BD, to develop a novel scoring model for predicting a severe course of disease, and to clarify the correlation between the DAIBD and endoscopic severity.

In the present study, we first sought to identify the endoscopic factors associated with disease activity for intestinal BD. On univariate analysis, the significant endoscopic predictors of disease activity were depth of intestinal ulcers, ulcer shape and ulcer size. Multivariate analysis revealed that the number of intestinal ulcers and volcano-shaped ulcers were independent predictors of the DAIBD. These results were consistent with those of several prior studies. A Korean study[15] reported that volcano-shaped ulcers responded least to medical treat-

ment, required surgery more frequently, and showed more frequent recurrences than geographic and aphthous-shaped ulcers. Another Korean study[16] revealed that individuals with deep ulcers and ulcers larger than 20 mm had an increased rate of steroid use and more frequent intestinal surgery. Moreover, a recent study by Jung *et al*[20] suggested that those with volcano-shaped ulcers demonstrated a less favorable response to surgical treatment, had more frequent recurrences, and more frequently required reoperation. In addition to volcano-shaped, deep, and larger than 20 mm ulcers, this study showed for the first time that having two or more intestinal ulcers was related to an increased disease severity when compared to solitary ulcers. Although there have been no reports regarding the correlations between the number of ulcers and disease activity in IBD, our results can be explained by the fact that an increase in the extent of mucosal involvement reflects the multiplicity of ulcers because patients with extensive and multiple ulcers are less likely to respond to local or systemic treatments. In systemic BD, the number, frequency, and healing time of oral ulcers and pain have been used to evaluate disease activity in clinical practice and studies[21]. In addition, in our study, ulcers in the ileocecal area showed a lower DAIBD score than ulcers in other location. A recent study on free bowel perforation in intestinal BD patients[22] reported that small bowel perforation was observed in 21.2%. Ileal ulcers might be at higher risk of complications such as perforation, which could be therefore related to severe disease activity. Further studies are required to validate whether the number of intestinal ulcers and ulcer location are truly associated with disease activity in intestinal BD.

Based on the predictive endoscopic factors for disease activity identified on multivariate analysis, we sought to develop an endoscopic severity model for intestinal BD. In multivariate analysis, besides the factors found to be significantly associated with the DAIBD in univariate analysis, potential factors that could achieve the optimized R^2 were also included, reflecting experts' suggestions. Initially, we hypothesized that endoscopic severity would have a highly significant correlation with clinical activity. However, the correlation between endoscopic severity and the DAIBD (r = 0.434) was unexpectedly found to be substantially weak. These results suggest that endoscopic severity might not correlate with clinical disease activity; thus, we cannot precisely judge a patient's condition using only the clinical activity index. Another potential reason for the lack of correlation may be that improvement in endoscopic lesions appeared later than the patient's clinical status. Although there have been no reports on correlations between endoscopic severity and disease activity in intestinal BD, many previous studies have described a poor association between endoscopic severity and clinical activity in IBD. In CD, the Groupe d'Etudes Therapeutiques des Affections Inflammatoires Digestives have shown that endoscopic severity patterns assessed by the CDEIS correlated poorly (r = 0.32) with clinical activity measured by the CDAI[23]. Daperno *et al*[24] indicated that the correlation of the simple endoscopic score for CD with CDAI (r = 0.39) was better than for the CDEIS, but still far from optimal. In contrast, Allez *et al*[25] observed that patients with CD exhibiting deep and extensive ulcerations on colonoscopy have a more aggressive clinical course with an increased rate of penetrating complications and increased likelihood of surgery. In UC, Carbonnel *et al*[26] demonstrated that extensive and deep ulcerations observed on colonoscopy were associated with an increased risk of colectomy, and another study by Daperno *et al*[27] reported that severe endoscopic lesions were significantly more frequent in non-responders to medical treatment compared with responders. These findings suggest that clinical disease activities might not highly correlate with concrete progress of patients. Endoscopic activity indexes could complement this drawback of clinical indices of IBD including intestinal BD. Further large-scale studies are needed to conclude whether endoscopic severity is superior to clinical disease activity in intestinal BD in terms of reflecting exactly the patient's current condition and predicting prognosis.

Because the correlation between endoscopic severity and the DAIBD was weak, recognizing reasons for the disagreement would be useful for clinicians in practice. To determine possible explanatory factors for the discrepancy between endoscopic severity and clinical activity, we considered laboratory results as well as clinical findings. These unexpected results might be explained that many systemic manifestations of the inflammatory process that result in the appearance of symptoms, but are not necessarily reflected in the mucosal lesions. Patients with systemic BD symptoms including oral and genital ulcers, eye and skin lesions, or arthralgia or concomitant infections may present with higher DAIBD scores than the predicted scores. Moreover, patients with predominant irritable bowel syndrome symptoms might present with higher clinical activity scores because symptoms of irritable bowel syndrome include nonspecific abdominal pain or discomfort and changes in stool frequency and appearance with the onset of symptoms, which might mimic IBD symptoms[28]. In the present study, patients with higher DAIBD scores than predicted scores had more irritable bowel syndrome symptoms and a lower rate of corticosteroid use. It is possible that clinical symptoms improve more quickly with the use of corticosteroids.

Moreover, in a group of patients with a higher endoscopic severity than actual clinical activity, we anticipated that serum biomarkers such as CRP and ESR would explain the discrepancy because the utility of laboratory markers for assessment of IBD activity, risk of complications, prediction of relapse and for monitoring the effect of therapy have been investigated. Consigny *et al*[29] reported that CRP levels (> 20 mg/dL) and ESR (> 15 mm/h) were predictive of relapse in CD, and Travis *et al*[30] revealed that elevated CRP (> 45 mg/L) could predict an

increased risk of colectomy in severe UC. Although many studies in the past few decades have noted that CRP and ESR are associated with systemic BD activity[31,32], the role of CRP and ESR in intestinal BD is still unknown. In the current study, the CRP and ESR levels were numerically higher in the severe predicted DAIBD group without any statistical difference. A recent study by Jung *et al*[33] also demonstrated that CRP or ESR levels were not highly correlated with disease activity in intestinal BD patients. Further studies are needed to evaluate the precise role of laboratory markers in intestinal BD.

The main strength of our study is that this is the first study to consider the use of endoscopy as a measurement tool of disease status in intestinal BD. However, because we retrospectively analyzed clinical and colonoscopic characteristics, our results might have been affected by the limitations of its retrospective design. In addition, the correlations between the predicted endoscopic severity model and the disease activity of intestinal BD were far below the expected values. Further studies to predict the clinical outcomes more accurately by taking into account endoscopic factors and the DAIBD together are required to verify our results.

In conclusion, we evaluated endoscopic factors related to disease activity of intestinal BD and the correlations between clinical activity and endoscopic severity. Our findings indicate that clinicians should closely observe patients with two or more intestinal ulcers and those with volcano-shaped ulcers and that these endoscopic findings could help clinicians make decisions regarding medical strategies for the treatment of intestinal BD. Finally, the understanding of discrepancy between actual and predicted DAIBD by the endoscopic severity model could be helpful in making an appropriate decision regarding medical strategies.

COMMENTS

Background

Because inflammatory bowel disease (IBD) has various, fluctuated clinical courses with repeated remission and relapse, the disease activity index is helpful for disease management. Moreover, recent studies suggested that mucosal healing was associated with a better outcome. Therefore, the macroscopic mucosal appearance is also considered a valuable indicator of disease activity. In this regard, disease activity index including endoscopic severity has been used in ulcerative colitis and Crohn's disease (CD).

Research frontiers

A study investigating a cohort of CD patients under long-term treatment with infliximab recently reported that mucosal healing was associated with a significantly lower need for abdominal surgery and data from the Active Ulcerative Colitis Trials 1 and 2 have suggested that mucosal healing was associated with a decreased risk of relapse. Moreover, because endoscopy could provide a direct assessment of the disease extent and severity, endoscopic severity has been estimated in IBD. However, although the disease activity index for intestinal Behcet's disease (DAIBD) was recently introduced, there are no specific tools for the measurement of endoscopic severity in intestinal Behcet's disease (BD).

Innovations and breakthroughs

This is the first study to consider the use of endoscopy as a measurement tool of disease status in intestinal BD. Their study demonstrated that the number of intestinal ulcers and volcano-shaped ulcers were predictive factors for severe DAIBD scores. However, the correlation between endoscopic severity and DAIBD (r = 0.434) was weak. Patients with severe DAIBD scores but with moderately predicted disease activity by the endoscopic severity model had more symptoms of irritable bowel syndrome and a lower rate of corticosteroid use than those with severe DAIBD scores and accurately predicted disease by the model.

Applications

This study suggests that clinicians should closely observe patients with two or more intestinal ulcers and those with volcano-shaped ulcers and that these endoscopic findings could help clinicians make decisions regarding medical strategies for the treatment of intestinal BD. Furthermore, the understanding of discrepancy between actual and predicted DAIBD by the endoscopic severity model could be helpful in making an appropriate decision regarding medical strategies.

Terminology

BD is a chronic, relapsing, multi-systemic inflammatory disorder and intestinal BD is diagnosed when patients with BD have predominant gastrointestinal symptoms and show intestinal ulcerations according to objective measures.

Peer review

In the present study, the authors showed that the number of intestinal ulcers and volcano-shaped ulcers were predictive factors for severe DAIBD scores, and that the correlation between endoscopic severity and DAIBD (r = 0.434) was weak. This study was well-investigated and will give us interesting information about the clinical indices of intestinal Behcet's disease.

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BRIEF ARTICLE

Bisphosphonate use and gastrointestinal tract cancer risk: Meta-analysis of observational studies

Yun Hwan Oh, Chan Yoon, Sang Min Park

Yun Hwan Oh, Sang Min Park, Department of Family Medicine, Seoul National University Hospital, Seoul National University College of Medicine, Seoul 110-774, South Korea
Chan Yoon, Seoul National University Hospital, Seoul National University College of Medicine, Seoul 110-774, South Korea
Author contributions: Oh YH and Yoon C contributed equally to this work; Yoon C was responsible for the study design, data acquisition, data extraction, data interpretation, statistical analysis and conducting the study; Oh YH was responsible for data acquisition, data extraction, data interpretation and conducting the study; and Park SM was responsible for the conception, study design, data interpretation, critical revision and supervising the study.
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Correspondence to: Sang Min Park, MD, MPH, PhD, Department of Family Medicine, Seoul National University Hospital, Seoul National University College of Medicine, 28 Yeongeon-Dong, Jongno-Gu, Seoul 110-774,
South Korea. smpark.snuh@gmail.com
Telephone: +82-2-20723331 Fax: +82-2-20723276
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Abstract

AIM: To perform a meta-analysis of observational studies to further elucidate the relationship between oral bisphosphonate use and gastrointestinal cancer risk.

METHODS: Systematic literature search was conducted in MEDLINE, EMBASE, and the Cochrane Library to identify studies through January 2011. Search terms were "bisphosphonates" or trade names of the drugs, and "observational studies" or "cohort studies" or "case-control studies". Two evaluators reviewed and selected articles on the basis of predetermined selection criteria as followed: (1) observational studies (case-control or cohort studies) on bisphosphonate use; (2) with at least 2 years of follow-up; and (3) reported data on the incidence of cancer diagnosis. The DerSimonian and Laird random effects model were used to calculate the pooled relative risk (RR) with 95% confidence interval (CI). Two-by-two contingency table was used to calculate the outcomes not suitable for meta-analysis. Subgroup meta-analyses were conducted for the type of cancer (esophageal, gastric and colorectal cancers). Sensitivity analyses were performed to examine the effect sizes when only studies with long-term follow-up (mean 5 years; subgroup 3 years) were included.

RESULTS: Of 740 screened articles, 3 cohort studies and 3 case-control studies were included in the analyses. At first, 4 cohort studies and 3 case-control studies were selected for the analyses but one cohort study was excluded because the cancer outcomes were not categorized by type of gastrointestinal cancer. More than 124 686 subjects participated in the 3 cohort studies. The mean follow-up time in all of the cohort studies combined was approximately 3.88 years. The 3 case-control studies reported 3070 esophageal cancer cases and 15 417 controls, 2018 gastric cancer cases and 10 007 controls, and 11 574 colorectal cancer cases and 53 955 controls. The percentage of study participants who used bisphosphonate was 2.8% among the cases and 2.9% among the controls. The meta-analysis of all the studies found no significant association between bisphosphonate use and gastrointestinal cancer. Also no statistically significant association was found in a meta-analysis of long-term follow-up studies. There was no negative association between bisphosphonate use and the incidence of esophageal cancer in the overall analysis (RR 0.96, 95% CI: 0.65-1.42, I^2 = 52.8%, P = 0.076) and no statistically significant association with long-term follow-up (RR 1.74, 95% CI: 0.97-3.10, I^2 = 58.8%, P = 0.119). No negative association was found in the studies reporting the risk of gastric cancer (RR 0.89, 95% CI: 0.71-1.13, I^2 = 0.0%, P = 0.472). In case of colorectal cancer, there was no association between colorectal cancer and bisphosphonate use (RR 0.62, 95% CI: 0.30-1.29, I^2 = 88.0%, P = 0.004) and

also in the analysis with long-term follow-up (RR 0.61, 95% CI: 0.28-1.35, I^2 = 84.6%, P = 0.011).

CONCLUSION: Oral bisphosphonate use had no significant effect on gastrointestinal cancer risk. However, this finding should be validated in randomized controlled trials with long-term follow-up.

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Key words: Bisphosphonate; Gastrointestinal tract cancer; Esophageal cancer; Gastric cancer; Colorectal cancer; Meta-analysis

Peer reviewer: Dr. Kok Sun Ho, Department of Colorectal Surgery, Singapore General Hospital, Outram Road, Singapore 169608, Singapore

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INTRODUCTION

Bisphosphonates are commonly used in postmenopausal women with osteoporosis. *In vitro* and *in vivo* studies have suggested that bisphosphonate has anticancer properties[1]: promoting apoptosis[2], inhibiting tumor cell adhesion and invasion[3], inhibiting angiogenesis[4], altering tumor-associated macrophage function[4], and enhancing immune surveillance[1,5]. However, it was recently reported that the Food and Drug Administration received reports of 23 cases of esophageal cancer in the United States and another 31 cases in Europe and Japan, all occurring from 1995 through 2008 among patients using oral bisphosphonates[6]. Since then, there have been several cohort studies and case-control studies to elucidate the association between the use of bisphosphonate and the risk of gastrointestinal tract cancer. However, results of the observational studies are inconsistent[7,8].

As yet, there have been no randomized controlled trials demonstrating a causal relationship between bisphosphonate use and gastrointestinal track cancer. Moreover, no meta-analysis has been performed despite the inconsistent results of observational studies. Therefore, in the present study, we aimed to investigate the association between the use of oral bisphosphonate and the risk of gastrointestinal cancer *via* meta-analysis of cohort studies and case-control studies.

MATERIALS AND METHODS

Study selection

We conducted a systematic literature search of MEDLINE, 1977 April 2011; EMBASE, 1971 April 2011; and the Cochrane Database of Systematic Reviews in the Cochrane Library, 1973 April 2011. We identified observational studies of bisphosphonate use whose primary or secondary outcomes included gastrointestinal tract cancer. The search terms were "bisphosphonates" or trade names of the drugs, and "observational studies" or "cohort studies" or "case-control studies" (Table 1). All the searches were restricted to human studies. In addition, a manual review of references from primary and review articles was performed to locate any additional relevant studies. All the potentially relevant articles were independently reviewed by 2 investigators (Oh YH and Yoon C). Disagreements between evaluators were resolved by discussion or consultation with a third author (Park SM).

The inclusion criteria were: (1) observational studies (case-control or cohort studies) on bisphosphonate use; (2) with at least 2 years of follow-up; and (3) reported data on the incidence of cancer diagnosis.

Data synthesis

To compute a pooled relative risk (RR) with 95% confidence interval (CI), we used the RRs or odds ratios and 95% CIs that were adjusted for most confounders. Because the incidence of cancer is generally low, we did not distinguish between the various measures of RR. If the outcome measures were unsuitable for meta-analysis, we calculated a crude estimate using a two-by-two contingency table.

We also assessed the heterogeneity for each meta-analysis by using the I^2 value which measures the percentage of total variation across that is attributable to heterogeneity rather than chance. High value of I^2 index suggests increased heterogeneity. We also calculated P value of Q-test which represents heterogeneity. If P value is less than 0.10, it represents there is heterogeneity.

Because of the known clinical and methodological heterogeneity of the studies we analyzed, we calculated the pooled RR with 95% CI based on the DerSimonian and Laird random effects model. We used Stata Version 11.0 (Stata Corp., College Station, Texas) for the statistical analysis.

Statistical analysis

Subgroup meta-analyses were carried out for the study design (cohort and case-control) and type of cancer. For the cancer subgroup analyses, esophageal, gastric and colorectal cancers were analyzed independently.

We also performed sensitivity analyses to examine the effect sizes when only studies with long-term follow-up (mean 5 years; subgroup 3 years) were included.

RESULTS

Identification of relevant studies

Figure 1 is a flow diagram of how we identified the relevant studies. Of the 740 articles identified, 4 cohort studies[7,9-11] and 3 case-control studies[8,12,13] were selected for the analyses. Many of initial 742 articles are not in

Table 1 Search strategy

Search strategy for MEDLINE	Search strategy for EMBASE	Search strategy for Cochrane reviews of the Cochrane Library
1. Diphosphonates [MH] OR diphosphonates [ALL] OR diphosphonate [ALL]	1. Bisphosphonates/de	1. Bisphosphonates [ALL]
2. Bisphosphonates [ALL]	2. Diphosphonate/de	2. Diphosphonate [ALL]
3. Alendronate [MH] OR alendronate [ALL]	3. Alendronate/de	3. Alendronate [ALL]
4. Clodronic acid [MH] OR clodronic acid [ALL] OR clodronate [ALL]	4. Clodronate/de	4. Clodronate [ALL]
5. Etidronic acid [MH] OR etidronic acid [ALL] OR etidronate [ALL]	5. Etidronate/de	5. Etidronate [ALL]
6. Ibandronic acid [NM] OR ibandronic acid [ALL] OR ibandronate [ALL]	6. Ibandronate/de	6. Ibandronate [ALL]
7. Minodronate [ALL] OR YM 529 [NM] OR YM 529 [ALL]	7. Minodronate/de	7. Minodronate [ALL]
8. Neridronate [ALL] OR 6-amino-1-hydroxyhexane-1,1-diphosphonate [NM] OR 6-amino-1-hydroxyhexane-1,1-diphosphonate [ALL]	8. Neridronate/de	8. Neridronate [ALL]
9. Olpadronic acid [NM] OR olpadronic acid [ALL] OR olpadronate [ALL]	9. Olpadronate/de	9. Olpadronate [ALL]
10. Pamidronate [NM] OR pamidronate [ALL]	10. Pamidronate/de	10. Pamidronate [ALL]
11. Risedronic acid [NM] OR risedronic acid [ALL] OR risedronate [ALL]	11. Risedronate/de	11. Risedronate [ALL]
12. Tiludronic acid [NM] OR tiludronic acid [ALL] OR tiludronate [ALL]	12. Tiludronate/de	12. Tiludronate [ALL]
13. Zoledronic acid [NM] OR zoledronic acid [ALL] OR zoledronate [ALL]	13. Zoledronate/de	13. Zoledronate [ALL]
14. 1 OR 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8 OR 9 OR 10 OR 11 OR 12 OR 13	14. 1 OR 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8 OR 9 OR 10 OR 11 OR 12 OR 13	14. 1 OR 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8 OR 9 OR 10 OR 11 OR 12 OR 13
15. Observational studies [ALL]	15. Observational studies/de	
16. CohORt studies [ALL]	16. CohORt studies/de	[ALL] - in all text
17. Case control studies [ALL] OR case-control studies [ALL]	17. Case control studies/de OR case-control studies/de	
18. Case referent studies [ALL] OR case-referent studies [ALL]	18. Case referent studies OR case-referent studies	
19. 15 OR 16 OR 17 OR 18	19. 15 OR 16 OR 17 OR 18	
20. 14 OR 19	20. 14 OR 19	
21. Humans [MH]	21. [Humans]/lim	
22. 20 AND 21	22. [Embase]/lim OR [embase classic]/lim	
	23. 20 AND 21 AND 22	
Date of search: April 29, 2011 (1973 April 2011); Result: 1709 articles found	Date of search: April 29, 2011 (1977 April 2011); Result: 2129 articles found	Limitation: cochrane database of systematic reviews; Date of search: April 29, 2011 (1971 April 2011); Result: 47 articles found

ALL: All fields; MH: MeSH terms; NM: Supplementary concept/substance name; PT: Publication type; /de: Mapped terms; /lim: Limitation.

our scope. They are mainly about effect of bisphosphonates, effects of several types of bisphosphonate regimen, other adverse effects of bisphosphonate, such as atrial fibrillation, osteonecrosis of jaw syndrome, and anti-cancerous bisphosphonate use for breast cancer and prostate cancer. We performed review of titles and abstract of the articles, then excluded irrelevant for our study. Thirty articles were remained after overall process, but 23 of 30 articles included no available data for outcome measures. One cohort study[9] was excluded because the cancer outcomes were not categorized by type of gastrointestinal cancer.

We also contacted Dr. Chris Cardwell to ask the data for the adjusted hazards ratio and 95% CI for gastric cancer only in bisphosphonate users[7].

Characteristics of the studies included in the analyses

More than 124 686 subjects participated in the 3 cohort studies[7,10,11] used in our meta-analyses. The mean follow-up time in all of the cohort studies combined was approximately 3.88 years (range: 0.5-13 years). The 3 case-control studies[8,12,13] used in our meta-analyses reported the number of cases and controls: 3070 esophageal cancer cases and 15 417 controls, 2018 gastric cancer cases and 10 007 controls, and 11 574 colorectal cancer cases and 53 955 controls. The percentage of study participants who used bisphosphonate was 2.8% among the cases and 2.9% among the controls. Tables 2 and 3 summarize the general characteristics of the studies included in our analyses.

The methods of the studies we included were assessed on the basis of 5 predetermined quality assessment items (Table 4). All of the studies included in our analyses were based on the secure record linkage regarding medication use and cancer diagnosis.

Association between bisphosphonate use and the risk of gastrointestinal tract cancer

There was no negative association between bisphosphonate use and the incidence of esophageal cancer in the overall analysis (RR 0.96, 95% CI: 0.65 1.42, I^2 = 52.8%, P = 0.076; Figure 2A). In 2 studies with long-term follow-up, there was a tendency for bisphosphonate users to develop esophageal cancer; however, this finding was not statistically significant (RR 1.74, 95% CI: 0.97-3.10, I^2 = 58.8%, P = 0.119). No significant association was

Table 2 Characteristics of cohort studies (n = 3) included in meta-analysis regarding use of bisphosphonates and risk of cancer

Ref.	Country	Cohort formation (population)	Study period	Mean F/U duration, yr (range)	Age, yr (mean ± SD)	Sex ratio (reference group)	Type of cancer	Type of drug (reference group)	Risk estimate	Adjustment	No. of cancer development/ no. of study population		No. of total cohort
											Bisphosphonate user	Reference group	
Abrahamsen *et al*[10]	Denmark	Patients with fracture who had prescription for oral bisphosphonate (national registers)	E:1995-2005; F/U: -2005	2.8 (NA)	74.3 ± 8.8	M: 11; F: 89	Esophageal cancer, gastric cancer	Any bisphosphonate use (no bisphosphonate use)	Esophageal or gastric cancer 0.78 (0.49-1.26); esophageal cancer 0.35 (0.14-0.85); gastric cancer 1.23 (0.68-2.22)	Age, sex, fracture type, Charlson index, number of concomitant medication, time to event	NA/13 678	NA/27 356	41 034
Solomon *et al*[11]	United States	Patients who had prescription for oral bisphosphonate (health care utilization records of medicare beneficiaries)	NA	NA (NA)	NA	NA	Esophageal cancer	Any bisphosphonate use (other osteoporosis medications use)	Esophageal cancer 0.55 (0.06-4.72)	NA	Incidence rate 26.7/100 000	Incidence rate 48.4/100 000	NA
Cardwell *et al*[7]	United Kingdom	Patients receiving prescription for oral bisphosphonate (GPRD)	E:1996-2006; F/U: -2008	4.5 (0.5-12.9)	70.0 ± 11.4	M: 19; F: 81	Esophageal cancer, gastric cancer	Any bisphosphonate use (regardless of bisphosphonate use)	Gastric cancer 0.78 (0.50-1.23); esophageal cancer 1.07 (0.77-1.49)	Age, sex, general practice, BMI, cigarette smoking, alcohol intake, hormone therapy, NSAID use, Barrett's esophagus, GERD, H2 receptor antagonist use, proton pump inhibitor use	Esophageal or gastric cancer 116/41 826 (gastric cancer 37/41 826; esophageal cancer 79/41 826)	Esophageal or gastric cancer 115/41 826 (gastric cancer 43/41 826; esophageal cancer 72/41 826)	83 652

E: Enrollment; F/U: Follow up; BMI: Body mass index; GERD: Gastroesophageal reflux disease; NSAID: Nonsteroidal antiinflammatory drugs; M: Male; F: Female; NA: Not available; GPRD: General Practice Research Database.

found in the studies[8,14] reporting the risk of gastric cancer (RR 0.89, 95% CI: 0.71-1.13, I^2 = 0.0%, P = 0.472; Figure 2B).

Bisphosphonate users were less likely to receive a diagnosis of colorectal cancer; however, this finding was not statistically significant in the overall analysis (RR 0.62, 95% CI: 0.30-1.29, I^2 = 88.0%, P = 0.004) or in the analysis limited to studies with long-term follow-up (RR 0.61, 95% CI: 0.28-1.35, I^2 = 84.6%, P = 0.011; Figure 2C).

DISCUSSION

Main findings

In the present study, we examined the association between oral bisphosphonate use and the incidence of gastrointestinal tract cancer by analyzing the results of previ-

Table 3 Case-control studies (n = 3) included in meta-analysis regarding use of bisphosphonates and risk of cancer

Ref.	Country (study type)	Case selection method	Control sampling method	Medication data collection method (period)	Age, yr (mean ± SD)	Sex ratio of cases (reference group)	Site of cancer	Type of drug (reference group)	Odd's ratio (95% CI)	Adjustment	No. of cases/no. of controls	
											Exposed	Unexposed
Green *et al*[8]	United Kingdom (nested case-control)	Review of computerized information (within participants of GPRD between 1995 -2005)	Matched on age (within 2 yr), sex, observation period in the database and general practice attended)	Review of computerized medical records (from 1995 until cancer diagnosis)	72 ± 11	M: 57; F: 43	Gastrointestinal cancer (esophageal, gastric, colorectal)	Any bisphosphonate use (no bisphosphonate use)	Esophageal cancer 1.30 (1.02-1.66); Gastric cancer 0.87 (0.64-1.19); colorectal cancer 0.87 (0.77-1.00)	Age, sex, observation period, general practice, BMI, cigarette smoking, alcohol intake	Esophageal cancer 90/345; gastric cancer 49/270; colorectal cancer 276/1555	Esophageal cancer 2864/14 376; gastric cancer 1969/9737; colorectal cancer 10 365/51 467
Nguyen *et al*[12]	United States (nested case-control)	Review of computerized information (within patients with Barrett's esophagus in the national veterans affair database between 2000-2002)	Matched on age(interval of 5 yr) and Barrett's esophagus index date	Review of computerized medical records (from Barrett's esophagus diagnosis until 3 mo before cancer diagnosis)	65.0 ± 10.3	M: 2.6; F: 97.4	Esophageal cancer	Any bisphosphonate use, mostly alendronate (no bisphosphonate use)	0.81 (0.18-3.72)	Age, Barrett's esophagus index date, race, non cancer disease comorbidity index, NSAID use, PPI use	2/13	114/683
Rennert *et al*[13]	Israel (conventional case-control)	Review of a computerized information (within postmenopausal women in CHS database between 2000-2006)	Matched on age, sex, residence, and ethnic group in CHS database	Review of CHS pharmacy records	71.1 ± NA	F: 100	Colorectal cancer	Any bisphosphonate use more than 1 yr (no bisphosphonate use)	0.41 (0.25-0.67)	BMI, family history of colorectal cancer, vegetable consumption, sports participation, use of low-dose aspirin, statins, vitamin D, postmenopausal hormones	53/100	880/833

E: Enrollment; F/U: Follow up; CI: Confidence interval; M: Male; F: Female; NA: Not available; BMI: Body mass index; NSAID: Nonsteroidal antiinflammatory drugs; PPI: Proton pump inhibitors; CHS: Clalit Health Service; GPRD: General Practice Research Database.

ous observational studies. Our meta-analyses found no significant association between the use of oral bisphosphonate and the overall gastrointestinal cancer incidence.

In the study design subgroup meta-analysis, no statistically significant association was found for the cohort studies or case-control studies. The cancer type subgroup analysis identified no association between bisphosphonate use and esophageal cancer or gastric cancer. Colorectal cancer was less likely to be diagnosed in bisphosphonate users; however, this finding was not statistically significant. The results of the sensitivity analyses of the studies with more than 3 years of oral bisphosphonate use were similar to those of the overall analyses.

According to our analyses, oral bisphosphonate seems to have little association with the incidence of gastrointestinal cancer.

It is well known that the use of oral bisphosphonate induces gastrointestinal problems, such as erosive esophagitis[15]. The endoscopic and histological findings of mucosal injury of the esophagus in patients using oral bisphosphonates[15-20] suggest that prolonged use may increase the risk of esophageal cancer. Green *et al*[8] analyzed the United

Table 4 Assessment of study quality

Study		Quality assessment items			
Representativeness	**Ascertainment of exposure: Secure record or structured interview**	**Demonstration that outcome of interest**		**Assessment of outcome: Independent blind assessment or record linkage was not present at start of study**	**Study controls for age, cigarette smoking, BMI status**
Cohort studies					
Steinbuch *et al*[9]	- (female)	+	+	+	- (randomized controlled trials)
Abrahamsen *et al*[10]	+	+	-	+	+ (age)
Solomon *et al*[11]	- (medicare beneficiaries)	+	-	+	- (not reported)
Cardwell *et al*[7]	+	+	+	+	+++ (age, cigarette smoking, BMI)
Case-control studies					
Green *et al*[8]	+	+	-	+	+++ (age, cigarette smoking, BMI)
Nguyen *et al*[12]	- (Barrett's esophagus)	+	+	+	+ (age)
Rennert *et al*[13]	- (female)	+	+	+	++ (age, BMI)

BMI: Body mass index.

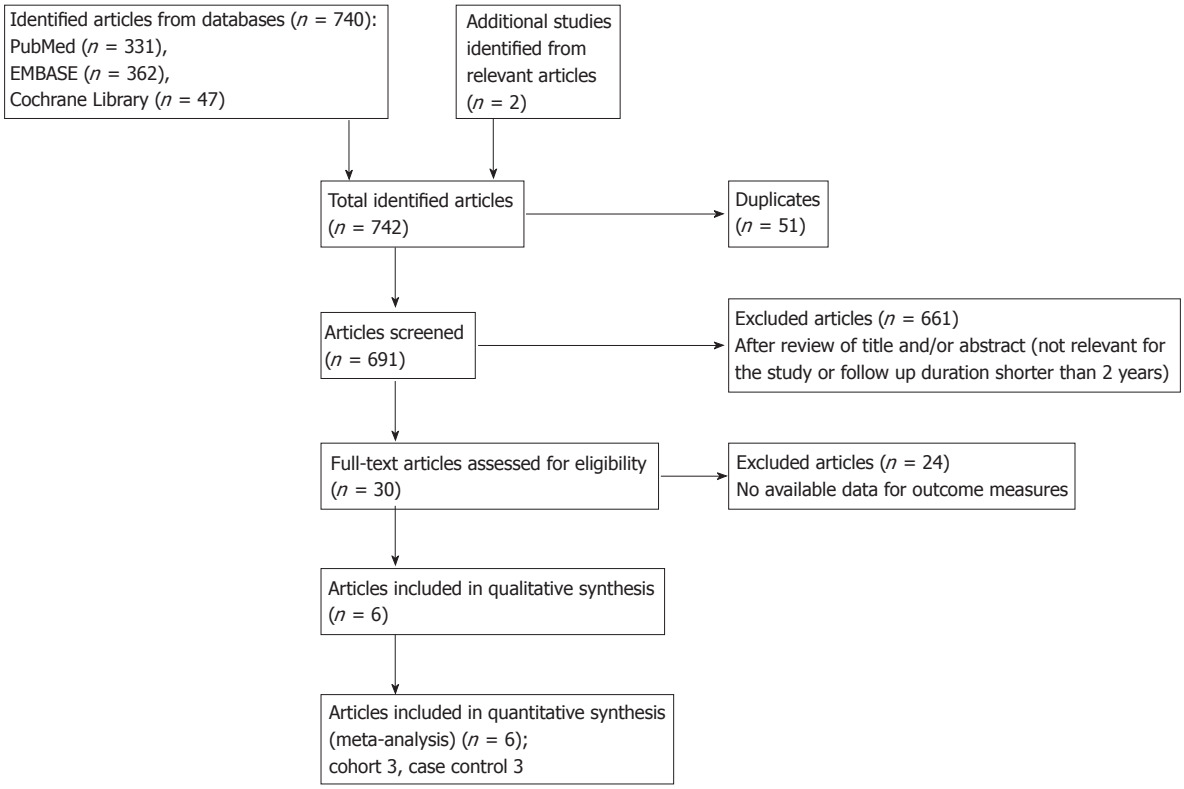


Figure 1 Flow diagram of the identification of the relevant cohort studies and case-control studies.

Kingdom General Practice Research Database and reported that bisphosphonate use increases esophageal cancer risk (RR 1.30, 95% CI: 1.02-1.66). The cancer-promoting effect was even greater in patients who used the drug for more than 3 years (RR 2.24, 95% CI: 1.47-3.43). We can infer from this result that bisphosphonate should be restricted among people with risk factors for esophageal cancer, such as Barrettv's esophagus. However, according to the study of Nguyen *et al*[12], the use of oral bisphosphonate does not increase the risk of esophageal adenocarcinoma in patients with Barrett's esophagus (incidence density ratio 0.92; 95% CI: 0.21-4.15). The risk of esophageal adenocarcinoma in patients with Barrett's esophagus is 30- to 125-fold greater than the risk in the general population[21]. The carcinogenic effect of oral bisphosphonate, including damage to the esophagus due to the toxicity of the drug itself and the effect of contact between the pill and the esophageal mucosa[15], may expedite the development of esophageal cancer in patients with Barrett's esophagus. However, a correlation between the risk of esophageal adenocarcinoma and use of oral bisphosphonate by patients with Barrett's esophagus is inconsistent with the concept of the so-called Barrett pathway.

Several *in vivo* and *in vitro* studies suggest that bisphosphonate has anticancerous properties[1-5]. Clinical studies also implicate the anticancerous effect of bisphosphonate in breast cancer[22]. It can be inferred from these

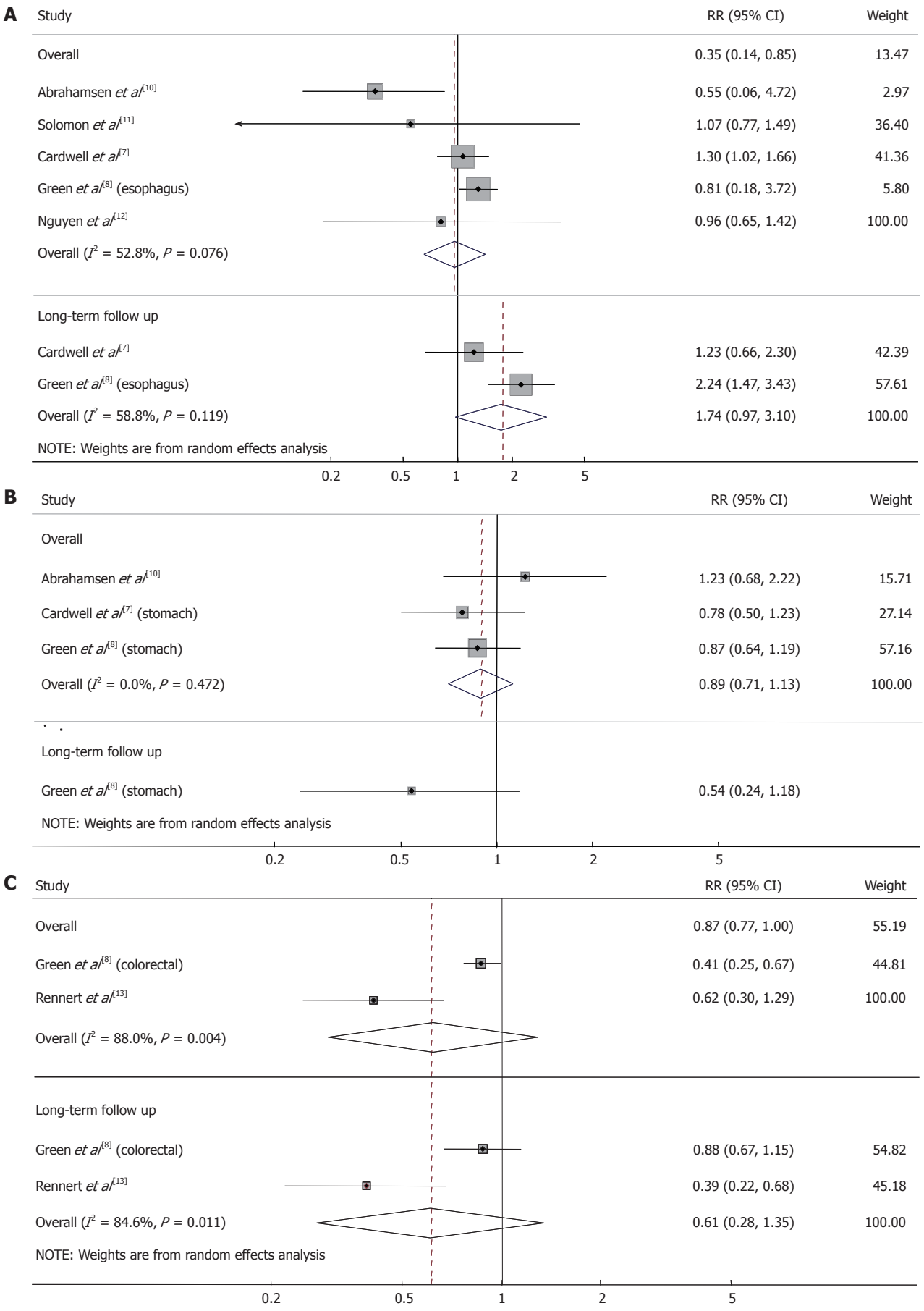


Figure 2 Association between bisphosphonate use and some cancer risk using the meta-analysis of the random effects model. A: Esophageal cancer risk; B: Gastric cancer risk; C: Colorectal cancer risk.

studies that there is no significant association between the use of oral bisphosphonate and the risk of esophageal cancer because bisphosphonate has anticancerous effects. As for procancerous effects, oral bisphosphonate directly irritates the esophageal mucosa and induces erosive esophagitis. On the contrary, bisphosphonate directly and indirectly interferes with cancer cell growth. Our finding about the correlation between the risk of esophageal cancer and oral bisphosphonate use suggests that the procancerous and anticancerous effects of bisphosphonate may cancel each other out.

There is also an implication about esophageal cancer within the statistical results in our study. Two observational studies that have great significance in our meta-analyses Caldwell *et al*[7] and Green *et al*[8], reported discrepant results (Figure 2A). According to the analyses of Dixon *et al*[23], the time-dependent RRs of esophageal cancer and oral bisphosphonate indicate no significant increased risk for esophageal cancer within 3 years of oral bisphosphonate use. However, when these investigators restricted their analyses to bisphosphonate use for more than 3 years, their results were different[23]. The Caldwell study[7] reported a RR of 1.01 (95% CI: 0.48-2.12); Green *et al*[8] reported a RR of 2.24 (95% CI: 1.47-3.43). The result of our meta-analysis was a RR of 1.74 (95% CI: 0.97-3.10) for the follow-up longer than 3 years. Our results show no statistical correlation between the long-term oral bisphosphonate use and the risk of esophageal cancer. However, in the study of Caldwell *et al*[7], the RR increased to 1.23 (95% CI: 0.66-2.3) when the analysis was restricted to a group of patients with a mean bisphosphonate use duration of 6.8 years. These results indicate that there is somewhat an association between prolonged oral bisphosphonate use and risk of esophageal cancer. Both studies used data from the Uinted Kingdom General Practice Research Database. Inconsistency between the 2 studies may be explained by differences in study design and confounding that could not be measured.

As stated previously, oral bisphosphonates induce esophagitis[15] and esophageal cancer that can be developed from reflux esophagitis through the Barrett pathway[19,20], and this is not a one-time process; it takes time. For this reason, recurrent injuries and healing processes induced by prolonged oral bisphosphonate use could be clinically meaningful factors. The long-term effect of oral bisphosphonate could be confirmed by an observational study that focuses on much longer periods of oral bisphosphonate use.

Regarding gastric cancer, our results show no significant association with oral bisphosphonate use. There is only 1 study that estimates RR dependent on duration of oral bisphosphonate use. Although there is no statistical significance, Green *et al*[8] found that RR is less for those using oral bisphosphonate longer than 3 years (RR 0.54, 95% CI: 0.24-1.18) than for those using oral bisphosphonate shorter than 3 years (RR 1.03, 95% CI: 0.67-1.59 for less than 1 year; RR 0.89, 95% CI: 0.52-1.53 for 1-3 years). This implies that there is no cumulative effect of oral bisphosphonate on the risk of gastric cancer. If bisphosphonate has competing procancerous and anticancerous properties, it may not affect the risk of cancer.

We performed a random-effects model analysis of the data on colorectal cancer because of high heterogeneity (I^2 = 88.0%, P = 0.004). According to our meta-analysis, oral bisphosphonate use demonstrates no effect on the risk of colorectal cancer (RR 0.62, 95% CI: 0.30-1.29). The analysis of the long-term use (more than 3 years) revealed that there is no statistically significant association between oral bisphosphonate use and colorectal cancer (RR 0.61, 95% CI: 0.28-1.35). However, the subgroup analysis shows a significant negative association between oral bisphosphonate use and the incidence of colorectal cancer (RR 0.87, 95% CI: 0.77-1.00; RR 0.41, 95% CI: 0.25-0.67). Therefore, it may be hasty to conclude on the basis of overall meta-analysis that oral bisphosphonate use does not affect colorectal cancer. In the study by Rennert *et al*[13], cases and controls were matched for age, ethnicity, family history of colorectal cancer, sports activity, vegetable consumption, body mass index (BMI), low-dose aspirin use, statin use, postmenopausal hormone use, calcium supplement use, and vitamin use, all of which can affect the risk of colorectal cancer. However, in Green *et al*[8], RRs were only adjusted for smoking status, alcohol intake and BMI. Thus, there were important differences in the study design and quality of methods.

The study by Rennert *et al*[13] implies that oral bisphosphonate has a protective effect against colorectal and breast cancers. The anticancerous effects of bisphosphonate, such as promoting apoptosis[2], inhibiting tumor cell adhesion and invasion[3], inhibiting angiogenesis[4], altering tumor-associated macrophage function[4], and enhancing immune surveillance, as previously mentioned, may have a key role in such a protective effect. One study reported that ibandronate reduces the incidence of colorectal dysplasia in mice with induced ulcerative colitis[24]. In colorectal cancer, different from esophageal cancer, oral bisphosphonate does not directly injure the intestinal mucosa or induce chronic mucosal inflammation and healing processes.

Considering this background, the results of our meta-analyses should be interpreted as inconclusive. A well-designed randomized controlled study or prospective cohort study is needed to confirm the preventive effect of oral bisphosphonate against colorectal cancer.

Our study has a few limitations. First, the number of studies we analyzed is small. There have been few studies about the correlation between oral bisphosphonate use and the risk for gastrointestinal cancer. For this reason, each study that reported on colorectal cancer reported a negative association, but the overall meta-analysis showed no statistical significance. Thus, oral bisphosphonate use is seemingly irrelevant to colorectal cancer. Second, the quality of our study depends on data from

original publications. Our study inevitably inherits some problems from the observational studies, such as selection bias, surveillance bias, and confounding. For example, more esophageal and gastric abnormalities might be observed in bisphosphonate users simply because they receive more endoscopic exams for abdominal discomfort caused by oral bisphosphonate.

We discussed the small number of studies regarding bisphosphonate use and development of gastrointestinal cancer in our article as a limitation. However, the actual number of overall subjects in our meta-analysis is not too small. In cohort studies, 124 686 subjects with mean follow up of 3.88 years were included in the final analysis. In case of case-control studies, there were 3070 esophageal cancer cases, 2018 gastric cancer cases and 11 574 colorectal cancer cases were included as well. Moreover, the results of two large observational studies were inconsistent. Caldwell *et al*[7] showed no significant association between bisphosphonate use and esophageal cancer. On the other hand, Green *et al*[8] had revealed the significant association. The inconsistent results of observational studies suggest the need of further studies as well as a meta-analysis.

Despite the negative results, our study is meaningful since it provides not an ultimate but a reasonable interim conclusion regarding the safety of bisphosphonate use before definite accumulation of long-term observational studies.

In summary, our meta-analyses indicate that there is no significant association between oral bisphosphonate use and the risk of gastrointestinal cancer. Oral bisphosphonate use has no significant association with the risk of esophageal cancer. There is an increased, though not statistically significant, risk of esophageal cancer in long-term users of oral bisphosphonate. An observational study focused on long-term use of bisphosphonate is needed to confirm this finding. The risk of gastric cancer is not associated with oral bisphosphonate use. Each study reporting on colorectal cancer indicates a negative association between the risk of colorectal cancer and oral bisphosphonate use, but our meta-analysis showed no statistically significant association. The confidence intervals were large (95% CI: 0.30-1.29). Thus, a randomized controlled trial or prospective cohort study should be performed to confirm the preventive effect of oral bisphosphonate.

COMMENTS

Background

There rises concerns about bisphosphonate use after the reports of 23 cases of esophageal cancer in the United States and another 31 cases in Europe and Japan, all occurring from 1995 through 2008 among patients using oral bisphosphonates. Bisphosphonate induced esophagitis, Barrett's esophagus and gastric ulcer can be the precancerous condition. There were observational studies to evaluate the risk between esophageal cancer and bisphosphonate use. But the results were inconsistent. So the overall evaluation of the association between gastrointestinal cancer and bisphosphonate is required.

Research frontiers

Meta-analysis was used to evaluate the risk of bisphosphonate for gastrointestinal cancer (esophageal, stomach, and colorectal cancer) in this study.

Innovations and breakthroughs

This meta-analysis systemically assessed the relation between bisphosphonate use and gastrointestinal cancer risk, and also showed site specific, long-term follow up results.

Applications

The results of meta-analysis in this study show that use of bisphosphonate has no significant association with gastrointestinal cancers. According to the results, bisphosphonate can be used without charge of carcinogen for now. But there should be a far more long-term observational studies to guarantee the long-term safety.

Terminology

Meta-analysis is method focused on contrasting and combining results from different studies to show the overall conclusion. It is essential component of a systematic review procedure.

Peer review

This is a good descriptive study in which authors perform a meta-analysis of observational studies to further elucidate the relationship between oral bisphosphonate use and gastrointestinal cancer risk. The results are interesting and suggest that oral bisphosphonate use had no significant effect on gastrointestinal cancer risk.

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BRIEF ARTICLE

Effect of ezetimibe on the prevalence of cholelithiasis

Assaf Stein, Doron Hermoni, Avishay Elis, Fred M Konikoff

Assaf Stein, Fred M Konikoff, Department of Gastroenterology and Hepatology, University of Tel Aviv, Meir Medical Center, Kfar Saba 44281, Israel
Doron Hermoni, Department of Family Medicine, Clalit Health Services Sharon, Netanya 42470, Israel
Avishay Elis, Department of Medicine A, University of Tel Aviv, Meir Medical Center, Kfar Saba 44281, Israel
Author contributions: Stein A performed the majority of the research and wrote the manuscript; Konikoff FM designed the research and supervised the work; Elis A was involved in data acquisition and editing the manuscript; Hermoni D participated in data acquisition and analysis.
Correspondence to: Dr. Assaf Stein, MD, Department of Gastroenterology and Hepatology, University of Tel Aviv, Meir Medical Center, 59 Tchernichovsky Street, Kfar Saba 44281, Israel. assaf.stein@clalit.org.il
Telephone: +972-9-7472580 Fax: +972-9-7472167
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Abstract

AIM: To investigate the prevalence of cholelithiasis among patients treated with ezetimibe.

METHODS: A retrospective, case-control study based on computerized medical records from patients of the Clalit Health Services, Sharon-Shomron region, from 2000 to 2009. Patients 20-85 years of age, who had been treated with ezetimibe and statins or statins only for at least 6 mo, and who had an abdominal ultrasound were included in the study. Collected data included age, gender, ezetimibe treatment duration, presence of hypothyroidism or diabetes, and existence of cholelithiasis as determined by ultrasound. Excluded were subjects after gallbladder resection, with hemolysis, myeloproliferative or inflammatory bowel diseases, and those treated with ursodeoxycholic acid and fibrates. Patients treated with statins and ezetimibe (study group) were compared to patients treated with statins only (control group).

RESULTS: The study group included 25 patients and the control group 168. All patients in the study were treated with statins. The study group included 13 males (52%) and 12 females (48%), the control group 76 males (45%) and 92 (55%) females (P = 0.544). The groups did not differ in age (mean age: 68 ± 8 years, range 53-85 years *vs* mean age: 71 ± 8 years, range 51-85 years; P = 0.153) or in the rate of diabetic and hypothyroid patients [11 (44%) *vs* 57 (33%), P = 0.347 in the study group and 5 (20%) *vs* 23 (14%), P = 0.449 in the control group, respectively]. Patients in the study group were treated with ezetimibe for an average of 798 ± 379 d. Cholelithiasis was found in 4 (16%) patients in the study group and in 33 (20%) patients in the control group (P = 0.666).

CONCLUSION: Ezetimibe does not appear to influence the prevalence of gallstones.

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Key words: Bile; Cholesterol; Neiman-Pick C1-like Receptor; Gallstones; Ezetimibe

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INTRODUCTION

Cholelithiasis is one of the most common diseases of the digestive system in the developed world. Its prevalence according to ultrasound-based studies is estimated to be 18.8% in females and 9.5% in males. Cholesterol stones make up 80%-90% of the gallstones in the Western world[1-8].

A delicate balance exists in the bile between the con-

centration of cholesterol and its main solubilizers, bile acids and phospholipids. Once the carrying capacity of cholesterol is exceeded, it can crystallize and initiate the formation of gallstones[5].

The treatment of hyperlipidemia consists of the inhibition of the two cholesterol sources in the body. Statins reduce cholesterol synthesis by inhibiting the key enzyme hydroxy methylglutaryl coenzyme A reductase, while ezetimibe decreases intestinal cholesterol absorption[9-12].

Ezetimibe blocks the mucosal Niemann-Pick C1-like1 receptor (NPC1L1)[12-18]. This causes a decrease in cholesterol absorption from the small intestine[16,19-22], loss of cholesterol into the stool and a decrease in the amount of cholesterol delivered to the liver and bile, thereby reducing the amount of cholesterol available for the formation of gallstones. Human liver cells also express the NPC1L1 receptor, which is located on the canalicular membrane and retains cholesterol by preventing its loss into bile and stool[12,13]. Thus, an opposite effect on bile is expected: biliary cholesterol enrichment and the formation of crystals and stones may occur when the receptor is inhibited by ezetimibe[12].

A few recent studies have evaluated the effects of hypocholesterolemic medication on gallstone formation. One study addressed the role of the hepatocyte NPC1L1 receptor and its function in cholesterol absorption from the biliary tract[12,13]. Transgenic mice with Hepatic NPC1L1receptor over-expression had a 10-30 fold decrease in biliary cholesterol without any effect on phospholipids or bile acid concentration. Biliary and plasma cholesterol concentrations returned to normal when ezetimibe was added[12]. In another study, Altmann *et al*[13] evaluated the effect of ezetimibe on gallstone formation in gallstone-susceptible mice. Ezetimibe prevented the formation of gallstones by effectively reducing intestinal cholesterol absorption. In the same study, 30 d of ezetimibe treatment was reported to decrease the biliary cholesterol concentrations and cholesterol saturation index in overweight patients with and without gallstones. Another study reported fewer cholecystectomies among subjects treated with statins compared to those not treated with the drug[14].

The aim of this study was to evaluate the prevalence of cholelithiasis among patients treated with ezetimibe. Since ezetimibe is usually not prescribed as monotherapy, we addressed the question in patients treated with concomitant statins.

MATERIALS AND METHODS

This retrospective case-control study was based on a review of computerized medical records of patients in the Sharon-Shomron region of Clalit Health Services (CHS), the largest health maintenance organization in Israel (and the second largest in the world), from 2000 to 2009.

The demographic and clinical data of all CHS members are contained in a central computer. This database is automatically updated with hospitalizations, invasive procedures, laboratory and imaging results, prescribed and purchased medications and mortality data.

The study inclusion criteria were patients 20 to 85 years of age, taking statins or statins and ezetimibe, with abdominal ultrasound results that included the presence or absence of gallstones. Statin or statin and ezetimibe treatment must have begun at least six months prior to the ultrasound. Subjects after gall bladder resection, with hemolysis, myeloproliferative or inflammatory bowel diseases, and those treated with ursodeoxycholic acid and fibrates were excluded.

The extracted data included age, gender, ezetimibe treatment duration, comorbidity with diabetes mellitus or hypothyroidism and the presence of gallstones based on abdominal ultrasound.

Patients were eligible for the study regardless of the indication for the ultrasound. In cases where a patient had more than one ultrasound, the most recent result was considered. All the scans were reviewed by an experienced radiologist.

Patients treated with ezetimibe and statins were compared to a matched control group of patients treated with statins only, to evaluate the prevalence of cholelithiasis.

The work was approved by the ethics committee of our institution (Helsinki Committee, Meir Medical Center, Israel, approval No. 020-09).

Statistical analysis

Data from the study groups were analyzed using the Pearson χ^2 test or the Fisher's exact test (each when appropriate) for non-parametric data and *t* test for continuous parametric data.

RESULTS

The study group included 25 patients treated with statins and ezetimibe. The control group consisted of 168 patients treated with statins only. Clinical and demographic data are presented in Table 1.

There were 13 males (52%) and 12 females (48%) in the study group and 76 males (45%) and 92 (55%) females ($P = 0.544$) in the control group. The groups did not differ in age (mean age: 68 ± 8 years, range 53-85 years *vs* 71 ± 8 years, range 51-85 years; $P = 0.153$) or in the rate of diabetic and hypothyroid patients [11 (44%) *vs* 57 (33%), $P = 0.347$ in the study group and 5 (20%) *vs* 23 (14%), $P = 0.449$ in the control group, respectively]. The mean duration of ezetimibe treatment was 798 ± 379 d. All patients were treated with statins. In the study group: simvastatin in 6 patients, atorvastatin 8, rosuvastatin 7 and pravastatin 4.

Cholelithiasis was found in 4 study group patients; 1 taking simvastatin and 3 treated with atorvastatin. In the control group: simvastatin 119, atorvastatin 19, rosuvastatin 11, and pravastatin 17 patients. Among the control group, 28 of the 33 gallstone positive patients had been

Table 1 Demographic and clinical characteristics of the patient groups

Study parameters	Study group (statins + ezetimibe) (n = 25)	Control group (statins only) (n = 168)	P value
Male/female	13 (52)/12 (48)	76 (46)/92 (55)	0.544
Mean age (yr)	68 ± 8	71 ± 8	0.153
Co-morbidities			
Diabetes	11 (44)	57 (34)	0.347
Hypothyroidism	2 (20)	23 (14)	0.449
Ezetimibe treatment			
Mean period (d)	799 ± 379		
Range (d)	183-1540		
Patients with gallstones	4 (16)	33 (20)	0.666

Data are presented as mean ± SD or n (%).

treated with simvastatin, 4 with atorvastatin, and 1 with rosuvastatin.

No statistically significant difference was found in the prevalence of gallstones in the study group (4, 16%) compared to the control group (30, 20%), $P = 0.666$.

No statistically significant differences were found in the prevalence of gallstones when controlling for age. Among patients ≤ 65, gallstones were present in 2 (18%) in the study group compared to 8 (17%) in the control group, $P = 0.619$. Among patients older than 65 years of age cholelithiasis was present in 2 (14%) study group patients and 25 (21%) control group patients, $P = 1$ by Fisher's exact test.

DISCUSSION

The results of this study show that treatment with ezetimibe in addition to statin therapy did not influence the prevalence of cholelithiasis. We compared patients treated with ezetimibe and statins to those treated with statins only because ezetimibe, which is not effective when used as monotherapy [low-density lipoprotein cholesterol (LDL-C) reduction of only about 16%], is usually given in combination with statins to patients who do not reach the recommended LDL-C levels with statin treatment (where it provides an additional reduction of 25% in LDL-C levels)[16,19-25].

The study was initiated because of two contradictory findings concerning the effect of ezetimibe on bile composition and gallstone formation[12,13]. Temel *et al*[12] addressed the inhibition of the hepatic canalicular membrane NPC1L1 receptor, which absorbs cholesterol from the bile. Inhibition of the receptor increases biliary cholesterol levels and the possibility of gallstone formation. In contrast, Altmann *et al*[13] reported that ezetimibe treatment resulted in decreased cholesterol bowel absorption and a net change in the bile composition to a less lithogenic type, when used in a mouse model as well as in human subjects[13].

To date, it is not clear whether statin-ezetimibe treatment over time leads to an increase or decrease in the prevalence of gallstones. A recent study revealed that statin-treated patients had fewer cholecystectomies compared to patients who did not take statins[14]. However, that study did not address the presence of gallstones or ezetimibe treatment[14]. Our study showed that the combination of statin and ezetimibe did not affect gallstone formation. A possible explanation could be the fact that statin treatment reduces the prevalence of gallstones, which masks the effect of ezetimibe on cholelithiasis.

The study has limitations, including the small number of patients who received ezetimibe and lack of information regarding the presence of gallstones prior to treatment with ezetimibe. However, this lack of information was true for both study groups. Another possible weakness might be the duration of ezetimibe treatment. Nevertheless, a mean duration of over two years seems a reasonable period in which to assess both gallstone formation and dissolution.

In conclusion, adding ezetimibe to statin therapy did not affect the prevalence of gallstones compared to treatment with statins alone.

ACKNOWLEDGMENTS

This study has been presented in abstract form at the Digestive Disease Week in Chicago May 8, 2011.

COMMENTS

Background

Hyperlipidemia treatment consists of the inhibition of the two cholesterol sources in the body. Statins reduce cholesterol synthesis by inhibiting the key enzyme hydroxy methylglutaryl coenzyme A reductase, while ezetimibe decreases intestinal cholesterol absorption. This study was initiated because of two contradictory findings concerning the effect of ezetimibe on bile composition and gallstone formation. To date, it is not clear whether statin-ezetimibe treatment over time leads to an increase or decrease in the prevalence of gallstones.

Research frontiers

When using a combination of a statin and ezetimibe for the treatment of hyperlipidemia, it is unclear whether statin-ezetimibe treatment leads to an increase or decrease in the prevalence of gallstones over time. In this study, the authors did not demonstrate a reduction in gallstone prevalence.

Innovations and breakthroughs

This study shows that ezetimibe in combination with a statin does not affect gallstone formation.

Applications

Despite promising data in animal models, ezetimibe seems to have no potential in treating human gallstone disease.

Peer review

This is a good descriptive study in which authors investigate the prevalence of cholelithiasis among patients treated with ezetimibe. It is a retrospective study with negative results, yet it does convey a logical reason for the study. Hence, it may be accepted as a short paper.

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BRIEF ARTICLE

Treatment of chronic hepatitis delta virus with peg-interferon and factors that predict sustained viral response

Shaikh Samiullah, Devrajani Bikharam, Nasreen

Shaikh Samiullah, Devrajani Bikharam, Nasreen, Department of Medicine, Liaquat University of Medical and Health Sciences, Jamshoro/Hyderabad 71000, Pakistan
Author contributions: Samiullah S proposed the study and is the guarantor; Samiullah S and Bikharam D performed research and wrote the first draft; Nasreen collected and analyzed the data; all authors contributed to the design and interpretation of the study and to further drafts.

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Correspondence to: Dr. Shaikh Samiullah, Associate Professor, Department of Medicine, Liaquat University of Medical and Health Sciences, Jamshoro/Hyderabad 71000, Pakistan. shaikhsamiullah@yahoo.com
Telephone: +92-300-3019233 Fax: +92-22-9213306
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Abstract

AIM: To observe the efficacy of peg-interferon in the treatment of hepatitis delta virus (HDV) and to identify the factors that would be predictive of the sustained viral response (SVR).

METHODS: This prospective study was conducted in Medical Unit Ⅳ of the Liaquat University of Medical and Health Sciences Hospital Jamshoro from June 2008 to September 2011. This study cohort included all patients of either sex who presented during this time with hepatitis B surface antigen positivity, hepatitis B virus DNA > 20 000 IU/mL, serum glutamic pyruvic transaminase (SGPT) > 2(upper limit of normal), HDV-RNA positivity with fibrosis stage ≥ 2. Informed consent was obtained from each of these individuals. Patients were diagnosed with hepatitis D on the basis of detectable viral antibodies and the presence of HDV-RNA in their serum. A liver biopsy was performed in all cases and fibrosis staging was performed in accordance with the METAVIR scoring system. All eligible patients were administered peg-interferon at a weekly dosage of 1.5 μg/kg body weight for 48 wk. HDV-RNA was assayed at the end of this treatment period and again at 24 wk later. A biochemical response was determined by a normalization of SGPT at the end of the treatment or during follow up. The end of treatment response was defined by a HDV-RNA negative status. A sustained virological response was defined by undetectable serum HDV-RNA at six months after the end of treatment.

RESULTS: Among the 277 patients enrolled in our present study, 238 completed a course of peg-interferon therapy of which 180 (75.6%) were male and 58 (24.4%) female. Biochemical responses were achieved in 122/238 (51.3%) patients. End of treatment responses were achieved in 71/238 (29.8%) cases. A SVR was achieved in 70 of these patients (29.4%). A strong association was found between the SVR and the end of treatment responses (P = 0.001), biochemical responses (P = 0.001) and the degree of fibrosis (P = 0.002).

CONCLUSION: Peg-interferon therapy can induce remission in nearly one third of patients harboring HDV.

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Key words: Hepatitis D; Peg-interferon; Biochemical response

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INTRODUCTION

Hepatitis delta virus (HDV) is a defective RNA virus that requires hepatitis B virus (HBV) to achieve replication. In long-term carriers of HBV, HDV infection can result in acute fulminant hepatitis or severe chronic hepatitis often progressing to cirrhosis and hepatocellular carcinoma[1]. Nearly 15-30 million people worldwide are infected with HDV[2]. Hepatitis D is dependent on hepatitis B for its prevalence. In Pakistan, the prevalence of hepatitis B is about 2.5% but in certain areas of Sindh province is much higher, in the range of 4.5% to 6.4%, with consequent increases in the prevalence of hepatitis D[3]. These areas are collectively referred to as the delta belt.

The introduction of a vaccination program for HBV, blood testing including that of pregnant women, the application of safety measures against blood born infections, the use of disposable syringes, and an increased public awareness of hepatitis B has markedly reduced the prevalence of this disease in many countries. However, hepatitis B is still a major health problem in several countries including Pakistan where HDV was found in 16.6% of hepatitis B carriers[4,5]. Indeed, a recently published study has detected HDV in 36.6% to 69.5% of hepatitis B patients in different parts of Pakistan[6].

The treatment options for HDV have remained disappointing over many years. Oral agents such as ribavirin, lamivudine, and famciclovir have been found to be ineffective against HDV[7]. The effectiveness of currently available and seemingly more potent oral antiviral drugs against hepatitis B such as adefovir, dipivoxil, entecavir, telbivudine and tenofovir is difficult to assess due to a lack of adequate clinical data[8]. The only medication which has proved effective to date against HDV infection is interferon. Up to 70% of affected patients can achieve biochemical responses using this drug, among which significant numbers achieve a virological response[9]. The efficacy of interferon based treatments is dose related, as a 12 mo treatment at 9 MU was found to be more effective than lower dose regimens. Unfortunately because of the dose-related side effects of standard interferon, patient compliance has tended to be very poor[10]. Recently however, peg-interferon, a product of the conjugation of interferon with polyethylene glycol has replaced standard interferon in clinical use because its prolonged half life allows for a single weekly dose to be administered. There are few previous studies of peg-interferon variant to date and it is difficult to draw any conclusions regarding its efficacy[11]. An evaluation of this drug in a large number of patients is therefore required and is also necessary to assess the factors that are predictive of a viral response in the treated patients.

The aim of our current study was to observe the efficacy of peg-interferon in the treatment of HDV and to evaluate the factors that are predictive of an sustained viral response (SVR) in the treated patients.

MATERIALS AND METHODS

Patients and clinical setting

This prospective study was conducted in Medical Unit Ⅳ of the Liaquat University of Medical and Health Sciences Hospital, Jamshoro, from June 2008 to September 2011. The patient cohort comprised consecutive cases of either sex who were hepatitis B surface antigen (HBsAg) positive, had HBV DNA > 20 000 IU/mL, a serum glutamic pyruvic transaminase (SGPT) > 2 (upper limit of normal), and were HDV-RNA positive with a fibrosis stage ⩾ 2. Informed consent was obtained from each patient and the study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. All of the subject patients were received from Hepatitis Prevention and Control Program, Sindh Chief Minister's initiative.

Exclusion criteria

We did not include patients below 18 or above 65 years age; with concomitant hepatitis C virus infection; fibrosis stage < 2 on liver biopsy; or with decompensated cirrhosis of the liver, i.e., history of ascites, variceal bleeding or hepatic encephalopathy. Also excluded were pregnant women or nursing mothers and cases of metabolic liver disease such as Wilson disease or hemochromatosis. Leukopenia (< 2500/mm^3), neutropenia (< 1000/mm^3), hemoglobin < 10 g/dL, and the presence of other severe diseases (cardiomyopathy, diabetes mellitus, arterial hypertension, neoplasia, neurologic diseases, depression and/or psychiatric disorders were additional exclusion criteria.

Methods

Patients were diagnosed with hepatitis D on the basis of antibodies and HDV-RNA in the serum. Patients in our cohort were also positive for HBsAg and had hepatitis B DNA levels > 20 000 IU/mL. Routine serological tests such as complete blood counts, liver function test, prothrombin time, SGPT, serum albumin, anti hepatitis D antibodies, HBsAg and an ultrasound of the abdomen were performed by the research laboratory of the Liaquat university of Medical and Health Sciences. Hepatitis D RNA and HBV-DNA tests were performed by the molecular laboratory of Liaquat university of Medical and Health Sciences. Liver biopsies were performed for all patients upon obtaining written consent and having fully explained the procedure to each individual. The biopsy procedure was performed in each case under ul-

Table 1 Baseline characteristics of patients (n = 238)

Continuous variables (mean ± SD)	
Age (yr)	29 ± 8.5
Hemoglobin (g/dL)	11.49 ± 2.45
The mean platelet count (10^3/mm^3)	145.6 ± 14.3
SGPT (IU/L)	134.18 ± 14.3
Hepatitis B DNA level (IU/mL)	155 × 10^3 ± 70.1 × 10^3
Categorical variable, frequency (%)	
Sex	
Male	180 (75.6)
Female	58 (24.4)
Stage of fibrosis	
Stage 2	172 (72.3)
Stage 3	44 (18.5)
Stage 4	22 (9.2)
Biochemical response	122 (51.3)
End of treatment	71 (29.8)
Sustained viral response	70 (29.4)

SGPT: Serum glutamic pyruvic transaminase.

trasound guidance with a 14 gauge tru-cut biopsy needle. The biopsy sample was considered adequate if it was greater than 10 mm in size and contained more than 5 portal tracts[12]. Biopsy samples were sent to a pathologist who had no knowledge of the clinical status of the patient. The staging of fibrosis was performed in accordance with the METAVIR scoring system as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; or F4, cirrhosis[13].

Treatment and follow up

All eligible patients were administered peg-interferon weekly at 1.5 μg/kg body weight for 48 wk as part of the Hepatitis Prevention and Control Program, Sindh Chief Minister's initiative. This program was initiated by the Government of Sindh province in Pakistan and provides free peg-interferon-base treatments to all affected patients. This program covers a population of 55.24 million in the Sindh province of Pakistan.

Patients were followed up every month to obtain complete blood picture and SGPT. All patients were evaluated for possible complications of interferon therapy. HDV-RNA was assayed at the end of treatment and at 24th wk later. A biochemical response was confirmed if there was a normalization of the SGPT levels at the end of treatment or during follow up. An end of treatment response was defined as a HDV-RNA negative result at the end of treatment. An SVR was defined as undetectable HDV-RNA in the serum at six months after the end of treatment. A non responder was defined as a patient with persistent detectable HDV-RNA at the end of treatment[14].

Continuous variables such as age, SGPT and platelet count were computed as the mean ± SD. Categorical variables such as sex, end of treatment response, biochemical response, SVR and stage of fibrosis were expressed as a percentage. Receiver operating characteristic (ROC) curve comparisons were performed between the end of treatment response, biochemical response, stage of fibrosis with SVR by calculating the area under the curve and 95% confidence intervals (CIs) as described by Hanley *et al*[15]. A P value of 0.05 was considered statistically significant. All data were processed on SPSS Version 16.

Table 2 Relation between biochemical response, end of treatment response and stage of fibrosis with sustained viral response n (%)

Variable	SVR achieved	SVR not achieved	P value
BR	60/122 (49.1)	62/122 (50.9)	0.001
ETR	70/71 (98.5)	1/71 (1.5)	0.001
Stage of fibrosis			0.002
Stage 2	39/172 (22.6)	133/172 (77.3)	
Stage 3	18/44 (40.9)	26/44 (59.09)	
Stage 4	13/22 (59)	9/22 (40.9)	

SVR: Sustained viral response; BR: Biochemical response; ETR: End of treatment response.

RESULTS

Of the 277 patients enrolled in this study, 238 completed the course of interferon therapy. Among the remaining 39 patients, 31 were excluded from the study because of minimal (stage 0 and 1) fibrosis and eight patients were dropped because of interferon-induced complications early in study period. Of the remaining 238 patients who were analyzed, 180 (75.6%) were male and 58 (24.4%) female. The mean age was 29.6 ± 8.5 years, hemoglobin (g/dL) was 11.49 ± 2.45, platelet count (10^3/mm^3) was 145.6 ± 14.3, and SGPT (IU/L) was 134.18 ± 14.3. Hepatitis B DNA level (IU/mL) 155.1 ± 70.1. Fibrosis stage 2 was present in 172 (72.3%), stage 3 in 44 (18.5%) and stage 4 in 22 (9.2%) patients. A biochemical response was achieved in 122/238 (51.3%) patients and not in 116 (48.7%) cases. End of treatment responses were achieved in 71/238(29.8%) cases and not in 167 (70.2%) patients. SVR was achieved in 70 (29.4%) patients.

The baseline characteristics of the patients in the study cohort are listed in Table 1. A strong association was found between SVR and end of treatment response (P = 0.001) as 70/71 (98.5%) patients who obtained an end of treatment response also achieved an SVR. The association between an SVR and the stage of fibrosis was also significant (P = 0.002) as 39/172 (22.6%) patients with fibrosis stage 2, 18/44 (40.9%) patients with fibrosis stage 3 and 13/22 (59%) patients with fibrosis stage 4 achieved SVR, and with the biochemical response (P = 0.001) as 60/122 (49.1%) of such patients also achieved SVR. Table 2 shows relation of end of treatment response, biochemical response and stage of fibrosis with SVR. ROC curve comparing the end of treatment response, biochemical response, stage of fibrosis and SVR is shown in Figure 1. The end of treatment response was found to be predictive of an SVR with a sensitivity of 98%, specificity of 94%, positive

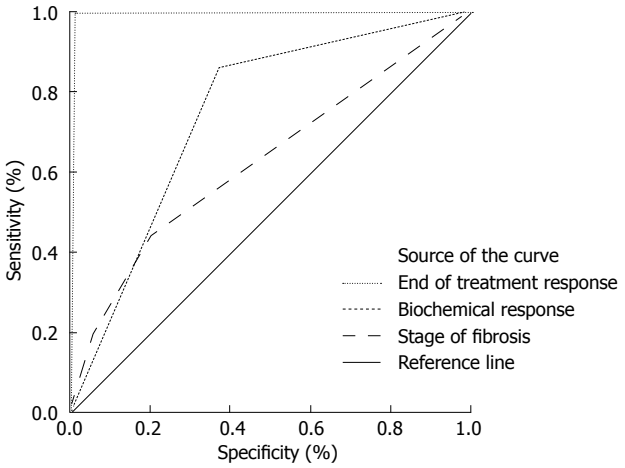


Figure 1 Receiver operating characteristic curve comparing end of treatment response, biochemical response, stage of fibrosis with sustained viral response. Receiver operating characteristic curve showing comparison between end of treatment response [area under curves (AUC) 0.997, 95% CI: 0.99-1.004], biochemical response (AUC 0.744, 95% CI: 0.67-0.81), stage of fibrosis (AUC 0.625, 95% CI: 0.54-0.70). The best predictor was end of treatment response at 99.7% (P = 0.001), followed by biochemical response 74.4% (P = 0.001) and stage of fibrosis 62.5% (P = 0.002).

predictive value (PPV) of 94.45, and negative predictive value (NPV) of 98% (P = 0.001). A biochemical response was also predictive of an SVR with a sensitivity of 85.7%, specificity of 63.2%, PPV of 69.9% and NPV of 81.5% (P = 0.001). The degree of fibrosis predicted an SVR with a sensitivity of 44.3%, specificity of 46%, PPV of 45% and NPV of 45.3% (P = 0.002).

DISCUSSION

This is the largest prospective study yet reported on the efficacy of peg-interferon monotherapy treatment for hepatitis D. The 238 patients included in the study cohort was far larger than the population analyzed in the previous hep-net-international delta hepatitis intervention trial, a multicenter trial comprising 90 patients in three arms. In our current prospective study, a biochemical response was achieved in 51.3%, end of treatment in 29.8% and SVR in 29.4% of the 238 patients with hepatitis D after 48 wk of peg-interferon therapy. Niro *et al*[16] previously studied 16 patients receiving peg-interferon for 72 wk and found biochemical response in 6 (37.5%) patients with 5 patients showing an end of treatment response and maintaining an SVR. In another earlier study by Wedemeyer *et al*[17] conducted on 29 patients receiving peg-interferon for 48 wk, a biochemical response was found in 8 (24%) cases and 9 (28%) patients showed an end of treatment response. When the therapy was extended to 72 wk, 13 (45%) patients achieved a complete biochemical response and 9 (31%) had an end of treatment response and SVR of 23% at 48 wk was improved to 28% at 72 wk. Erhardt *et al*[18] have reported that among 12 patients treated using peg-interferon, biochemical normalization occurred in 3 (25%) patients and an SVR in 2 (17%) patients. Castelnau *et al*[19] in their study of 12 patients treated for 12 mo with peg-interferon observed complete biochemical response in 8 (57%) patients, and an end of treatment response in 8 (57%) cases of whom 6 (43%) obtained an SVR. The discrepancies between the results of these various studies are likely due to the small number of patients analyzed.

In our present study, strong predictors of an SVR were an end of treatment response, biochemical response and the stage of fibrosis. We observed a strong association between the end of treatment response (P = 0.001) and an SVR as 70/71 (98.5%) patients achieved both responses. Indeed, an end of treatment response was predictive of an SVR with a PPV of 94.45% and an NPV of 98% (P = 0.001). Niro *et al*[16] also confirmed the importance of an end of treatment response as the 3/16 (19%) of their patients who achieved end of treatment response increased to 4/16 (25%) at the 24 wk follow up point. Wedemeyer *et al*[17] observed an end of treatment response in 9/29 (31%) of the patients in their cohort after 72 wk of treatment, out of which 5 patients maintained clearance of HDV-RNA at the end of follow up. However, the outcomes of the remaining four patients was unknown, making the end of treatment response an important predictor of SVR or end of follow up response.

Erhardt *et al*[18], in a study of 10 patients, found end of treatment response to peg-interferon in 2/10 (17%) cases that were maintained after 24 wk of follow up. Castelnau *et al*[19], in a study of 14 hepatitis D patients, report an SVR in all 8 (57%) patients who achieved an end of treatment virological response.

Based on our current findings, the biochemical response is an important predictor of an SVR as 60/70 (85.7%) (P = 0.001) of our hepatitis D patients achieved both responses. Niro *et al*[16] previously reported a biochemical response in 6 patients out of which 3 achieved SVR during a follow up of 11 patients for 72 wk. According to the study of Wedemeyer *et al*[17], 7 hepatitis D patients achieved SVR out of 13 (53.8%) patients who achieved a biochemical response after 72 wk of peg-interferon treatment. Castelnau *et al*[19] have reported that 75% of hepatitis D patients who achieved an end of treatment response were HDV-RNA negative six months after of the discontinuation of their treatment.

In our current analyses, the response to treatment increased with the degree of fibrosis (P = 0.002) as 39/172 (22.6%) patients with fibrosis stage 2, 18/44 (40.9%) patients with fibrosis stage 3 and 13/22 (59%) patients with fibrosis stage 4 achieved SVR. Reports on hepatitis D are sparse at present but the two publications recently presented at the 62nd Annual Meeting of the American Association for the Study of Liver Disease (2011) in San Francisco on hepatitis B has shed some new light on the importance of the stage of fibrosis for the treatment response outcome. Marcellin *et al*[20] treated 641 hepatitis B patients with tenofovir for five years and performed paired biopsies in 348 of these cases, one at baseline and a second at 5 years after the start of

treatment. These authors found an overall histological improvement in 98% of their patients, which was more marked in patients with advanced fibrosis. In another study by Gane *et al*[21], paired biopsies were performed in 72 Asian patients with hepatitis B receiving tenofovir treatment for five years. A histological regression of fibrosis of ⩾ 1 point decrease in the Ishak score was noted in 42/72 (58.3%) patients at five years which was more marked in 6/18 patients with liver cirrhosis. These two observations and our present findings indicate that the response to treatment will increase as the degree of fibrosis increases. This result will be of definite benefit in deciding the time of treatment.

Niro *et al*[16] in his study performed a baseline biopsy in 11 of 16 hepatitis D patients, of which 6 patients consented for paired biopsy after 72 wk of peg-interferon treatment. These authors reported an improvement of more than 1 point in Necro inflammatory activity as well as fibrosis in 4 out of 6 patients. Erhardt *et al*[18] report in their study of 12 patients that 11 allowed a baseline biopsy and 7 a followed biopsy after six months of treatment. An improvement in the stage of fibrosis was observed in responders as well as in non-responders. In contrast to previous studies however, no improvement was found in cases of cirrhosis of the liver.

In conclusion, peg-interferon therapy can induce remission in nearly one third of patients infected with HDV. Patients who achieve a biochemical response or an end of treatment response with a high stage fibrosis on liver histology, also achieved SVR.

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COMMENTS

Background

Nearly 150 million people worldwide are infected with hepatitis delta virus (HDV). Hepatitis D is dependent on hepatitis B for its prevalence. In Pakistan, the prevalence of hepatitis B is about 2.5% but in certain areas of Sindh province is much higher, in the range of 4.5% to 6.4%, with consequent increases in the prevalence of hepatitis D.

Research frontiers

The treatment options for HDV have remained disappointing over many years. Recently however, peg-interferon, a product of the conjugation of interferon with polyethylene glycol has replaced standard interferon in clinical use because its prolonged half life allows for a single weekly dose to be administered. Although the previous studies has shown sustained viral response (SVR) in substantial number of patients but the number of patient studied were so small that it was very difficult to draw any conclusion.

Innovations and breakthroughs

The above mentioned studies are on such small scale that's it is difficult to draw any conclusion. An evaluation of this drug in a large number of patients is therefore required and is also necessary to assess the factors that are predictive of a viral response in the treated patients. This is the largest prospective study yet reported on the efficacy of peg-interferon monotherapy treatment for hepatitis D. The 238 patients included in the study cohort was far larger than the population analyzed in the previous hep-net-international delta hepatitis intervention trial, a multicenter trial comprising 90 patients in three arms. In the authors' current prospective study, a biochemical response was achieved in 51.3%, end of treatment in 29.8% and SVR in 29.4% of the 238 patients with hepatitis D after 48 wk of peg-interferon therapy.

Applications

The present study has been conducted on larger scale. One of very important observation seen was better response of patients with more aggressive disease. This will further open the debate as this finding is in contrast to what was previously being observed that milder disease respond better. The present study will help with selecting the patients in future at proper time as well as those who can better response to treatment.

Peer review

This is a large study of 277 patients with delta virus infection. A total of 238 patients completed a full course of 48 wk of treatment with pegylated interferon. The SVR rate was 29.4%. The data is interesting.

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BRIEF ARTICLE

Endoscopic submucosal dissection for foregut neuroendocrine tumors: An initial study

Quan-Lin Li, Yi-Qun Zhang, Wei-Feng Chen, Mei-Dong Xu, Yun-Shi Zhong, Li-Li Ma, Wen-Zheng Qin, Jian-Wei Hu, Ming-Yan Cai, Li-Qing Yao, Ping-Hong Zhou

Quan-Lin Li, Yi-Qun Zhang, Wei-Feng Chen, Mei-Dong Xu, Yun-Shi Zhong, Li-Li Ma, Wen-Zheng Qin, Jian-Wei Hu, Ming-Yan Cai, Li-Qing Yao, Ping-Hong Zhou, Endoscopy Center and Endoscopy Research Institute, Zhongshan Hospital, Fudan University, Shanghai 200032, China

Author contributions: Li QL, Zhang YQ and Chen WF contributed equally to this work; Li QL, Yao LQ and Zhou PH conceived and designed this study; Zhou PH, Zhang YQ, Chen WF, Xu MD, Zhong YS, Ma LL, Qin WZ and Hu JW provided the study materials or patients; Li QL, Zhang YQ and Chen WF collected and assessed the data; Li QL, Zhang YQ and Cai MY writed the manuscript; all authors approved the manuscript.

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Correspondence to: Ping-Hong Zhou, MD, Endoscopy Center and Endoscopy Research Institute, Zhongshan Hospital, Fudan University, 180 FengLin Road, Shanghai 200032, China. zhou1968@yahoo.cn
Telephone: +86-21-64041990 Fax: +86-21-64038472

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Abstract

AIM: To evaluate the feasibility and efficacy of endoscopic submucosal dissection (ESD) for foregut neuroendocrine tumors (NETs).

METHODS: From April 2008 to December 2010, patients with confirmed histological diagnosis of foregut NETs were included. None had regional lymph node enlargement or distant metastases to the liver or lung on preoperative computerized tomography scanning or endoscopic ultrasonography (EUS). ESD was attempted under general anesthesia. After making several marking dots around the lesion, a mixture solution was injected into the submucosa. The mucosa was incised outside the marking dots. Dissection of the submucosal layer beneath the tumor was performed under direct vision to achieve complete *en bloc* resection of the specimen. Tumor features, clinicopathological characteristics, complete resection rate, and complications were evaluated. Foregut NETs were graded as G1, G2, or G3 on the basis of proliferative activity by mitotic count or Ki-67 index. All patients underwent regular follow-up to evaluate for any local recurrence or distant metastasis.

RESULTS: Those treated by ESD included 24 patients with 29 foregut NETs. The locations of the 29 lesions are as follows: esophagus (n = 1), cardia (n = 1), stomach (n = 23), and duodenal bulb (n = 4). All lesions were found incidentally during routine upper gastrointestinal endoscopy for other indications, and none had symptoms of carcinoid syndrome. Preoperative EUS showed that all tumors were confined to the submucosa. Among the 24 gastric lesions, 16 lesions in 11 patients were type I gastric NETs arising in chronic atrophic gastritis with hypergastrinemia, while the other 8 solitary lesions were type Ⅲ because of absence of atrophic gastritis in these cases. All of the tumors were removed in an *en bloc* fashion. The average maximum diameter of the lesions was 9.4 mm (range: 2-30 mm), and the procedure time was 20.3 min (range: 10-45 min). According to the World Health Organization 2010 classification, histological evaluation determined that 26 lesions were NET-G1, 2 gastric lesions were NET-G2, and 1 esophageal lesion was neuroendocrine carcinoma (NEC). Complete resection was achieved in 28 lesions (28/29, 96.6%), and all of them were confined to the submucosa in histopathologic assessment with no lymphovascular invasion. The remaining patient with NEC underwent additional surgery because the resected specimens revealed angiolymphatic and muscularis invasion, as well as incomplete

resection. Delayed bleeding occurred in 1 case 3 d after ESD, which was managed by endoscopic treatment. There were no procedure-related perforations. During a mean follow-up period of 24.4 mo (range: 12-48 mo), local recurrence occurred in only 1 patient 7 mo after initial ESD. This patient successfully underwent repeat ESD. Metastasis to lymph nodes or distal organs was not observed in any patient. No patients died during the study period.

CONCLUSION: ESD appears to be a safe, feasible, and effective procedure for providing accurate histopathological evaluations and curative treatment for eligible foregut NETs.

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Key words: Endoscopic submucosal dissection; Neuroendocrine tumor; Foregut

Peer reviewers: Masahiro Tajika, MD, PhD, Department of Endoscopy, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan; Giovanni D De Palma, Professor, Department of Surgery and Advanced Technologies, University of Naples Federico Ⅱ, School of Medicine, 80131 Naples, Italy; Yuji Naito, Professor, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

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INTRODUCTION

Depending on the point of origin in the disseminated endocrine system, gastrointestinal neuroendocrine tumors (NETs) can be classified as a tumor of the foregut (esophagus, stomach and duodenum), midgut (distal ileum and proximal colon), or hindgut (distal colon and rectum). Foregut NETs are considered rare, and the reported incidence is 10%-30% of all gastrointestinal NETs, but their incidence has been increasing in most countries over the last 50 years[1]. These increases in diagnosed incidence and prevalence are likely attributable, in part, to better awareness and improved diagnostic strategies regarding NETs, as well as the increasingly widespread use of upper gastrointestinal endoscopy[2].

Foregut NETs can show a broad range of clinical behavior, ranging from benign and asymptomatic to disseminated and metastatic. This clinical behavior is a reflection of tumor location, type, grade and stage. With the advent of screening gastroscopy, we currently can diagnose and treat small foregut NETs at a very early stage. Foregut NETs that are limited to the mucosa/submucosa and are less than 11-20 mm in size demonstrate a low frequency of lymph node and distant metastasis, and thus have been managed with local excision (including endoscopic treatment), which offers improved quality of life compared with surgery[3,4]. Nowadays traditional polypectomy and endoscopic mucosal resection (EMR) are most commonly employed for some foregut NETs[5-12]. However, complete histological resection may not always be easy to achieve by using EMR because most gastrointestinal NETs are not confined to the mucosa but, rather, invade the submucosa[13], which results in frequent involvement of the resection margin.

Endoscopic submucosal dissection (ESD) is a method of endoscopic resection that involves circumferential cutting of the mucosa surrounding the tumor followed by dissection of the submucosa under the lesion. ESD has the advantage of a high probability of *en bloc* and histologically complete resection even in huge lesions because the technique involves dissection of the submucosal tissue beneath the lesion[14-16]. To date, the fact that ESD can facilitate a histologically complete resection of NETs has been verified for the use of ESD for treatment of rectal carcinoid tumors (now referred to as NETs)[17-19]. However, no systematic data have yet been published in which ESD has been applied to foregut NETs. Thus, we retrospectively evaluated the feasibility and efficacy of ESD for foregut NETs in this study.

MATERIALS AND METHODS

Patients

With the approval of the institutional review board, 24 patients with confirmed histological diagnosis of foregut neuroendocrine neoplasms were treated with ESD from April 2008 to December 2010. None had regional lymph node enlargement or distant metastases to the liver or lung on computerized tomography (CT) scanning or endoscopic ultrasonography (EUS) before ESD. Tumor characteristics, complete resection rate, complications, local recurrences, and distant metastases were evaluated in all patients. Informed patient consent was obtained prior to the procedures. The procedures were in accordance with the Helsinki Declaration (1964, and amended in 1975, 1983, 1989, 1996 and 2000) of the World Medical Association.

ESD procedures

Preoperative EUS (high-frequency miniprobe, UM-2R, 12 MHz; UM-3R, 20 MHz, Olympus) was performed to evaluate the depth of tumor invasion and the involvement of regional lymph nodes. The existence of lymph node and distant metastasis was surveyed by contrast-enhanced CT, abdominal ultrasound, and chest X-ray.

To dissect the tumor, ESD was attempted with a single-channel gastroscope (GIF-H260, Olympus) and an insulated-tip electrosurgical knife (KD-611L, Olympus) or hook knife (KD-620LR, Olympus). A transparent cap (D-201-11304, Olympus) was attached to the tip of the gastroscope to provide direct views of the submucosal layer. Other equipment included an injection needle

Table 1 World Health Organization 2010 classification and suggested grading of neuroendocrine neoplasms of the digestive system[20]

Classification	Grading		
	Grade	Mitotic count (per 10 HPF)	Ki-67 index (%)
NET	G1	< 2	< 2
NET	G2	2–20	3-20
NEC	G3	> 20	> 20

HPF: High-power field; NEC: Neuroendocrine carcinoma; NET: Neuroendocrine tumor; G1: Low-grade tumors; G2: Intermediate-grade tumors; G3: High-grade tumors.

(NM-4L-1, Olympus), grasping forceps (FG-8U-1, Olympus), snare (SD-230U-20, Olympus), hot biopsy forceps (FD-410LR, Olympus), clips (HX-610-90, HX-600-135, Olympus), high-frequency generator (ICC-200, ERBE), and argon plasma coagulation unit (APC300, ERBE).

Patients were treated under general anesthesia. After making several marking dots with argon plasma coagulation around the lesion, a mixture solution (including 100 mL normal saline, 1 mL indigo carmine, and 1 mL epinephrine) was injected into the submucosa. The mucosa was incised outside the marking dots. Direct dissection of the submucosal layer beneath the tumor was then performed under direct vision to achieve a complete *en bloc* resection of the specimen. The tumor was dissected along the capsule, and saline solution was injected repeatedly during the dissection when necessary. The resultant artificial ulcer was managed routinely with argon plasma coagulation to prevent delayed bleeding and hemoclips were used to close the deeply dissected areas as needed.

Pathological evaluation

The World Health Organization (WHO) 2010 classification of tumors of the digestive system was used for histopathologic evaluation[20] (Table 1). Immunohistochemistry with the two robust neuroendocrine markers chromogranin A and synaptophysin was used to reach an accurate diagnosis. The mitotic count per 10 high-power fields or the Ki-67 index per 400 cells-2000 cells was used for grading and staging. On the basis of proliferative activity, foregut neuroendocrine neoplasms were graded as G1, G2 or G3. Low to intermediate grade tumors (G1-G2) were defined as NETs (previously referred to as carcinoids) whereas high-grade carcinomas (G3) were termed neuroendocrine carcinomas (NECs).

en bloc resection refers to a resection in one piece. A resection with a tumor-free margin in which both the lateral and basal margins were free of tumor cells was considered as a complete resection. A resection was considered as an incomplete resection in which the tumor extended into the lateral or basal margin, or the margins were indeterminate because of artificial burn effects.

Types of gastric NET

There is a clinicopathological categorization of gastric NETs which distinguishes the four types of neuroendocrine neoplasms of the stomach[3]: type Ⅰ are those arising in chronic atrophic gastritis with hypergastrinemia; type Ⅱ occurs in patients with hypergastrinemia due to Zollinger-Ellison syndrome in association with multiple endocrine neoplasia type Ⅰ; type Ⅲ are gastric NETs not associated with any specific pathogenetic background; and poorly differentiated NECs are nowadays classified as type Ⅳ neuroendocrine neoplasms of the stomach.

Indications for additional surgery

Additional surgical intervention was recommended in the case of type Ⅰ or type Ⅱ gastric NETs with positive margins, size > 20 mm, G2-G3 histological grading, invasion into the muscularis propria, or vessel infiltration of tumor cells. Additional surgery was also recommended in the case of type Ⅲ gastric NETs with a size > 10 mm irrespective of other risk factors. Surgery was the only treatment of choice in case of a localized type Ⅳ gastric NET.

Surgical indication for esophageal and duodenal NETs is controversial. The indication used in our study corresponded with that of type Ⅲ gastric NETs.

Follow-up

Patients underwent follow-up endoscopy and/or EUS at 1, 3, 6 and 12 mo after ESD and annually thereafter to view the wound healing and evaluate for any residual tumor or recurrence. Close follow-up was also carried out to evaluate for distant metastasis every 6 mo by abdominal ultrasound, contrast-enhanced CT, and chest radiography.

RESULTS

Patient characteristics, lesion features, and clinical outcomes are summarized in Table 2. The study cohort consisted of 9 men (ages 35 to 71 years) and 15 women (ages 35 to 82 years). Of these, 3 cases had 2 tumors and 1 case had 3 tumors (case nos. 12, 14, 20 and 24). The 29 lesions were found in the esophagus (n = 1), gastric cardia (n = 1), stomach (n = 23), or duodenal bulb (n = 4; Figure 1).

All lesions were found incidentally during routine upper gastrointestinal endoscopy for other indications such as anemia, reflux symptoms, or nonspecific abdominal symptoms. None had symptoms of carcinoid syndrome. With respect to macroscopic appearance, 15 patients had submucosal tumors with a central depression or erosion on top, 8 patients had sessile polyps with a reddened surface, and 1 patient had an erosion-type tumor.

EUS showed that all tumors were confined to the submucosa. Before ESD procedures, histological diagnosis of foregut NETs had been confirmed via biopsies in 4 cases (case nos. 8, 13, 15 and 22), suspected diagnosis of NETs were made based upon specific macroscopic appearances and EUS characteristics in 15 cases, and an indefinite diagnosis was made in 5 cases (case nos. 5, 7, 16, 18 and 23).

Table 2 Clinicopathological characteristics of 24 patients with foregut neuroendocrine tumors treated by endoscopic submucosal dissection

Case no.	Age (yr)	Gender	Location	Macroscopic appearance	Clinico-pathological type	Size (mm)	*en bloc* resection	Depth of invasion	WHO classification	Lateral margin	Vertical margin	Vessel invasion	Complication	Additional surgery	Follow-up (mo)
1	41	M	Gastric antrum	Submucosal tumor	Ⅲ	10	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	48
2	53	M	Gastric body	Submucosal tumor	I	5	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	40
3	71	M	Gastric body	Submucosal tumor	Ⅲ	18	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	Reject	36
4	55	F	Gastric body	Submucosal tumor	Ⅲ	10	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	30
5	82	F	Gastric fundus	Submucosal tumor	Ⅲ	30	Yes	Submucosa	NET-G2	(-)	(-)	Absent	None	Reject	30
6	35	F	Gastric fundus	Polyp	I	6	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	30
7	35	M	Duodenal bulb	Submucosal tumor	NA	15	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	Reject	30
8	57	M	Duodenal bulb	Submucosal tumor	NA	5	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	30
9	40	F	Gastric body	Polyp	I	6	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	30
10	47	F	Gastric body	Submucosal tumor	I	4	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	30
11	71	F	Duodenal bulb	Submucosal tumor	NA	6	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	24
12	56	F	Gastric body	Polyp	I	3, 2	Yes	Mucosa	NET-G1	(-)	(-)	Absent	None	None	24
13	51	F	Gastric body	Submucosal tumor	Ⅲ	8	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	24
14	55	M	Gastric body	Polyp	I	8, 3, 3	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	24
15	55	M	Gastric body	Polyp	I	4	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	18
16	45	F	Esophagus	Submucosal tumor	NA	30	Yes	Muscularis	NEC	(-)	(+)	Present	None	Yes	18
17	57	M	Gastric body	Submucosal tumor	Ⅲ	15	Yes	Submucosa	NET-G1	(-)	(-)	Absent	Delayed bleeding	Reject	22
18	35	F	Gastric body	Submucosal tumor	Ⅲ	25	Yes	Submucosa	NET-G2	(-)	(-)	Absent	None	Yes	18
19	70	F	Duodenal bulb	Submucosal tumor	NA	10	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	18
20	45	F	Gastric body	Polyp	I	6, 3	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	12
21	48	F	Gastric body	Polyp	I	5	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	12
22	55	M	Gastric body	Polyp	I	5	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	12
23	65	F	Cardia	Submucosal tumor	Ⅲ	20	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	Reject	14
24	35	F	Gastric fundus	Erosion	I	5, 3	Yes	Submucosa	NET-G1	(-)	(-)	Absent	None	None	12

"Reject" means the patient rejected the additional surgery. NA: Not applicable; NEC: Neuroendocrine carcinoma; NET: Neuroendocrine tumor; M: Male; F: Female; WHO: World Health Organization.

Among the 24 gastric lesions, 16 lesions in 11 patients were type I gastric NETs arising in chronic atrophic gastritis with hypergastrinemia, while the other 8 solitary lesions were type Ⅲ because of absence of atrophic gastritis. No patient had metastatic disease to lymph nodes or distal organs on preoperative examinations.

All of the tumors were removed in an *en bloc* fashion (29/29, 100%). The average maximum diameter of the lesions was 9.4 mm (range: 2-30 mm), and the average procedure time was 20.3 min (range: 10-45 min) (Figure 2A-H).

According to the WHO 2010 classification, results of pathological studies determined that 26 lesions were NET-G1, 2 gastric lesions were NET-G2 (case nos. 5 and 18), and 1 esophageal lesion was NEC (case no. 16). In the esophageal lesion defined as NEC, the tumor invaded the muscularis propria and the vertical margin was affected by tumor cells. Lymphatic and vascular invasion were also observed in the resected specimens. This

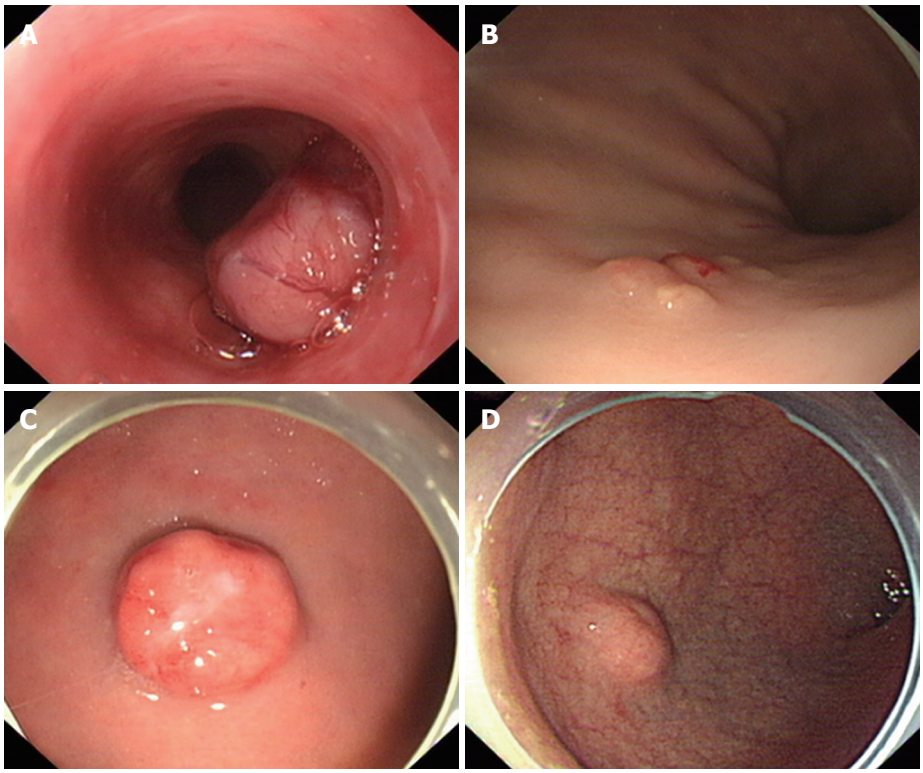


Figure 1 Macroscopic appearance of foregut neuroendocrine tumors in representative cases. A: An esophageal neuroendocrine tumor (NET; case no. 16); B: A type Ⅰ gastric NET (case no. 14); C: A type Ⅲ gastric NET (case no. 17); D: A duodenal NET (case no. 11).

patient thus underwent esophagectomy and excision of regional lymph nodes. Complete resection was achieved for the remaining 28 lesions (28/29, 96.6%), all of which were confined to the submucosa without lymphovascular invasion upon histopathologic assessment (Figures 2I-K).

Delayed bleeding occurred in 1 case 3 d after ESD (case no. 17). Successful hemostasis was achieved by coagulating forceps and spraying with thrombin during emergency endoscopy, and no blood transfusion was necessary. There were no procedure-related perforations.

Additional surgical intervention was considered in 6 cases (case nos. 3, 5, 7, 17, 18 and 23) because duodenal and type Ⅲ gastric NETs with a diameter larger than 10 mm may have a high risk of metastasis. However, only 1 of the 6 underwent additional surgery (case no. 18), and we could not reveal residual lesions or metastatic lymph nodes in the surgical specimens. The remaining 5 cases refused additional surgery, citing their age, physical condition, or other personal reasons. These patients remained under careful follow-up.

During a mean follow-up period of 24.4 mo (range: 12-48 mo), local recurrence occurred in only 1 patient 7 mo after initial ESD (case no. 14). This patient underwent successful repeat ESD. Metastasis to lymph nodes or distal organs was not observed in any patient, and no patient died during the study period.

DISCUSSION

Though rare, primary foregut NETs may now be discovered more often with the advent of screening gastroscopy[1]. Nowadays more and more foregut NETs are usually diagnosed at an early stage (tumor size < 11-20 mm and limited to the mucosa/submucosa)[1,3], and thus can be managed with local excision (including endoscopic treatment) because of a low frequency of lymph node and distant metastasis.

As a minimally invasive technique, endoscopic resection may benefit patients diagnosed with foregut NETs. This approach offers the promise of localized treatment of these tumors with relatively few complications and low mortality. Various endoscopic resection procedures have been described as potential treatment procedures for foregut NETs, such as endoscopic polypectomy, strip biopsy, aspiration resection, and band-snare resection[5-12]. However, complete resection of NETs is difficult with conventional polypectomy because most gastrointestinal NETs are not confined to the mucosa but, rather, invade the submucosa[13], which results in frequent involvement of the resection margin. As such, polypectomy may not provide adequate resection margins and additional surgical intervention may be needed.

In the current study we applied ESD to remove foregut NETs. Most of the lesions extended into the submucosa (96.6%, 28/29), and *en bloc* resection was achieved for all of the tumors in this study. Moreover, a histologically complete resection was achieved for 96.6% (28/29) of the current series. This is important as a high rate of histologically complete resections with ESD may give several advantages for the treatment of foregut NETs[17-19]. First, a histologically complete resection

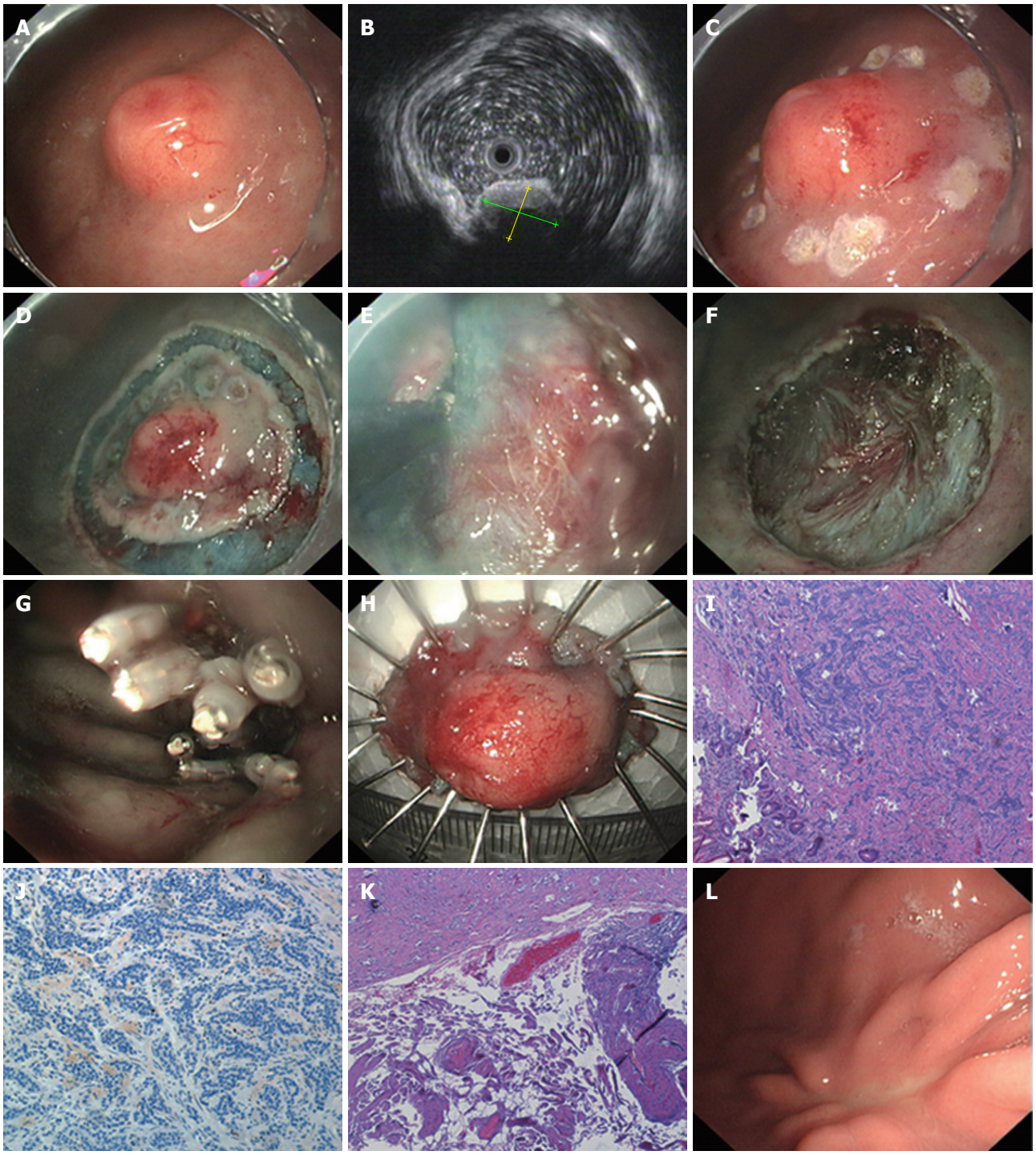


Figure 2 Endoscopic submucosal dissection of a type Ⅲ gastric neuroendocrine tumor in a 71 year-old man (case no. 3). A: A submucosal tumor with a central depression of the gastric body; B: Endoscopic ultrasonography shows a mass invading the submucosa (maximum diameter, 18 mm); C-G: Processes of endoscopic submucosal dissection with the use of a soft transparent hood; H: Completely resected specimen; I: Microscopic examination of the completely resected specimen reveals a neuroendocrine tumor (NET) within the submucosa of the stomach (hematoxylin-eosin staining, × 40); J: The lesion was NET-G1 on the basis of proliferative activity (Ki-67 index < 2%, × 100); K: The basal margin was free of tumor invasion (hematoxylin-eosin staining, × 40); L: Endoscopic findings of scar 12 mo after endoscopic submucosal dissection.

can provide a substantial amount of submucosal tissue, such that an accurate determination of lymphovascular invasion and histological grading is possible, which can inform decisions regarding subsequent therapy. Second, incomplete resection of tumors results in the need for additional surgery, and complete resection for a reduced frequency of unnecessary surgery. Third, repeat endoscopic resection of remnant tumor after an initial incomplete endoscopic resection may be difficult because of fibrosis that prevents lifting the lesion by submucosal injection. Therefore, we recommend histologically complete resection of foregut NETs even when lesions are

small, and the present study indicates that ESD may maximize the likelihood of such an outcome because of complete resection.

Bleeding and perforation are the two main complications of ESD. In this study, only 1 case had delayed bleeding 3 d after ESD. Successful hemostasis was achieved by coagulating forceps and spraying with thrombin during emergency endoscopy. No patient had immediate or delayed perforation. The relatively low ESD complication rate most likely reflects the small size of the lesions. Furthermore, we focus heavily on preventing and handling bleeding during the procedure because hemostasis may take a long time to establish and the endoscopic view may be affected. This is important because blind hemostasis may eventually lead to perforation. In our study, immediate minor bleeding was treated successfully by grasping the bleeding vessels with hot biopsy forceps and coagulating them during ESD. Direct coagulation with the hook knife was done for small vessels in the submucosa, and metallic clips were often deployed for more brisk bleeding.

Certain properties of foregut NETs should be considered for rescue surgery after endoscopic resection. The indication for this is usually based on the location, type, grade, and stage of the foregut NET disease. Considering the review of Scherübl *et al*[3] for the treatment of gastric NETs, in this study additional surgical intervention was recommended in the case of type I or type II gastric NETs with positive margins, size > 20 mm, G2-G3 histological grading, invasion into the muscularis propria, or vessel infiltration of tumor cells. Additional surgery was also recommended in the case of type III gastric NETs with a size > 10 mm irrespective of other risk factors. Surgery was the only treatment of choice in case of a localized type IV gastric NET. As for the endoscopic treatment of esophageal and duodenal NETs, the indication is controversial. In correspondence to a systemic review[4], additional surgery was recommended in our study in patients with duodenal NETs larger than 10 mm. According to these indications, additional surgical intervention should have been undertaken 7 cases; however, 5 of them refused additional surgery, citing their age, physical condition, or other personal reasons. Nonetheless, during a 2-year follow-up period, local recurrence or distal metastasis didn't occur in these 5 patients.

Thus, the appropriate selection criteria of foregut NETs for endoscopic resection is still controversial and is in need of further investigation. Because of complete resection, the present study indicates that ESD may reasonably serve as a curative treatment for foregut NETs when lesions are within the existing criteria. ESD also provides enough histological information for tumor grading and staging even when lesions are beyond the selection criteria, which informs decisions regarding subsequent surgery. In addition, endoscopic treatment might also be considered in particular in patients with a high risk of perioperative complications due to old age or advanced comorbidity, for example, or if there are other contraindications to major surgery, even though the lesions are a little beyond the existing criteria. If EUS is able to rule out invasion of the muscularis propria, the upper size limit for the lesion would only be restricted by what is endoscopically practicable[4]. If endoscopy is deemed unsuitable, laparoscopic techniques could be another attractive alternative[21,22].

In conclusion, ESD may be considered for the treatment of eligible foregut NETs because the technique shows a high histologically complete resection rate, provides accurate histopathological evaluation, has a low complication rate, and can be performed within a reasonable timeframe. Issues to be addressed in future prospective studies include identification of appropriate selection criteria and analysis of long-term results after ESD treatment of foregut NETs.

COMMENTS

Background

With the advent of screening gastroscopy, foregut neuroendocrine tumors (NETs) can be diagnosed at a very early stage and thus managed with local excision, including endoscopic treatment. However, complete resection of NETs is difficult with conventional polypectomy because most gastrointestinal NETs are not confined to the mucosa but, rather, invade the submucosa, which results in frequent involvement of the resection margin.

Research frontiers

Endoscopic submucosal dissection (ESD) is a method of endoscopic resection that involves circumferential cutting of the mucosa surrounding the tumor followed by dissection of the submucosa under the lesion. ESD has the advantage of a high probability of *en bloc* and histologically complete resection even in huge lesions because the technique involves dissection of the submucosal tissue beneath the lesion. To date, the fact that ESD can facilitate histologically complete resection of NETs has been verified on the use of ESD for the treatment of rectal carcinoid tumors (now referred to as NETs). However, no systematic studies have yet been published in which ESD has been applied for foregut NETs.

Innovations and breakthroughs

In the current study, the authors reported for the first time that ESD can reasonably serve as curative treatment for eligible foregut NETs when lesions are within the existing criteria. ESD also provides enough histological information for tumor grading and staging even when the lesions are beyond the selection criteria, which informs decisions regarding subsequent surgery.

Applications

The study suggests that ESD should be considered for treatment of eligible foregut NETs. Issues to be addressed in future prospective studies include identification of appropriate selection criteria and analysis of long-term results after ESD treatment of foregut NETs.

Terminology

Gastrointestinal NETs can be classified as tumor of the foregut (esophagus, stomach, and duodenum), midgut (distal ileum and proximal colon), or hindgut (distal colon and rectum).

Peer review

This is a good descriptive study reporting the results of endoscopic treatment for the resection of foregut NETs. The results are interesting and suggest that ESD may be considered for treatment of eligible foregut NETs because the technique shows a high histologically complete resection rate, provides accurate histopathological evaluation, has a low complication rate, and can be performed within a reasonable timeframe.

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BRIEF ARTICLE

Anorectal functional outcome after repeated transanal endoscopic microsurgery

Hong-Wei Zhang, Xiao-Dong Han, Yu Wang, Pin Zhang, Zhi-Ming Jin

Hong-Wei Zhang, Xiao-Dong Han, Yu Wang, Pin Zhang, Zhi-Ming Jin, Department of General Surgery, the Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai 200233, China
Author contributions: Zhang HW, Han XD, Wang Y, Zhang P, Jin ZM designed and performed the research; Zhang HW, Han XD and Jin ZM analyzed the data; Zhang HW and Jin ZM prepared the paper.
Supported by The Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai, China
Correspondence to: Dr. Zhi-Ming Jin, Department of General Surgery, the Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai 200233, China. zhw00sub@gmail.com
Telephone: +86-21-64369181 Fax: +86-21-64367326
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Abstract

AIM: To evaluate the status of anorectal function after repeated transanal endoscopic microsurgery (TEM).

METHODS: Twenty-one patients undergoing subtotal colectomy with ileorectal anastomosis were included. There were more than 5 large (> 1 cm) polyps in the remaining rectum (range: 6-20 cm from the anal edge). All patients, 19 with villous adenomas and 2 with low-grade adenocarcinomas, underwent TEM with submucosal endoscopic excision at least twice between 2005 and 2011. Anorectal manometry and a questionnaire about incontinence were carried out at week 1 before operation, and at weeks 2 and 3 and 6 mo after the last operation. Anal resting pressure, maximum squeeze pressure, maximum tolerable volume (MTV) and rectoanal inhibitory reflexes (RAIR) were recorded. The integrity and thickness of the internal anal sphincter (IAS) and external anal sphincter (EAS) were also evaluated by endoanal ultrasonography. We determined the physical and mental health status with SF-36 score to assess the effect of multiple TEM on patient quality of life (QoL).

RESULTS: All patients answered the questionnaire. Apart from negative RAIR in 4 patients, all of the anorectal manometric values in the 21 patients were normal before operation. Mean anal resting pressure decreased from 38 ± 5 mmHg to 19 ± 3 mmHg (38 ± 5 mmHg *vs* 19 ± 3 mmHg, $P = 0.000$) and MTV from 165 ± 19 mL to 60 ± 11 mL (165 ± 19 mL *vs* 60 ± 11 mL, $P = 0.000$) at month 3 after surgery. Anal resting pressure and MTV were 37 ± 5 mmHg (38 ± 5 mmHg *vs* 37 ± 5 mmHg, $P = 0.057$) and 159 ± 19 mL (165 ± 19 mL *vs* 159 ± 19 mL, $P = 0.071$), respectively, at month 6 after TEM. Maximal squeeze pressure decreased from 171 ± 19 mmHg to 62 ± 12 mmHg (171 ± 19 mmHg *vs* 62 ± 12 mmHg, $P = 0.000$) at week 2 after operation, and returned to normal values by postoperative month 3 (171 ± 19 *vs* 166 ± 18, $P = 0.051$). RAIR were absent in 4 patients preoperatively and in 12 ($\chi^2 = 4.947$, $P = 0.026$) patients at month 3 after surgery. RAIR was absent only in 5 patients at postoperative month 6 ($\chi^2 = 0.141$, $P = 0.707$). Endosonography demonstrated that IAS disruption occurred in 8 patients, and 6 patients had temporary incontinence to flatus that was normalized by postoperative month 3. IAS thickness decreased from 1.9 ± 0.6 mm preoperatively to 1.3 ± 0.4 mm (1.9 ± 0.6 mm *vs* 1.3 ± 0.4 mm, $P = 0.000$) at postoperative month 3 and increased to 1.8 ± 0.5 mm (1.9 ± 0.6 mm *vs* 1.8 ± 0.5 mm, $P = 0.239$) at postoperative month 6. EAS thickness decreased from 3.7 ± 0.6 mm preoperatively to 3.5 ± 0.3 mm (3.7 ± 0.6 mm *vs* 3.5 ± 0.3 mm, $P = 0.510$) at month 3 and then increased to 3.6 ± 0.4 mm (3.7 ± 0.6 mm *vs* 3.6 ± 0.4 mm, $P = 0.123$) at month 6 after operation. Most patients had frequent stools per day and relatively high Wexner scores in a short time period. While actual fecal incontinence was exceptional, episodes of soiling were reported by 3 patients. With regard to the QoL, the physical and mental health status scores (SF-36) were 56.1 and 46.2 (50 in the general population), respectively.

CONCLUSION: The anorectal function after repeated TEM is preserved. Multiple TEM procedures are useful for resection of multi-polyps in the remaining rectum.

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Key words: Familial adenomatous polyposis; Repeated transanal endoscopic microsurgery; Anorectal function; Anorectal manometry; Subtotal colectomy

Peer reviewers: Michael Leitman, MD, FACS, Chief of General Surgery, Beth Israel Medical Center, 10 Union Square East, Suite 2M, New York, NY 10003, United States; Patricia Sylla, MD, Assistant Professor of Surgery, Division of General and Colorectal Surgery, Massachusetts General Hospital, WACC 460, 15 Parkman Street, Boston, MA 02114, United States; Dr. Marek Bebenek, MD, PhD, Department of Surgical Oncology, Regional Comprehensive Cancer Center, pl. Hirszfelda 12, 53-413 Wroclaw, Poland

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INTRODUCTION

Familial adenomatous polyposis (FAP) is an autosomal dominant disease that is classically characterized by the development of hundreds to thousands of adenomas in the rectum and colon during the second decade of life. Almost all patients will develop colorectal cancer if they are not identified and treated at an early stage. The incidence of FAP at birth is approximately 1/8300[1] in both men and women, and less than 1% of them develop colorectal cancer[1].

Twenty years ago, many patients with FAP underwent subtotal colectomy in China. Currently, some of the older patients refused to undergo a complicated operation (such as ileal pouch-anal anastomosis) because of old age or poor physical status. However, polyps are still present in the remaining rectum of patients and some of these polyps are too large to be extirpated by a colonoscope. These large polyps often cause bleeding and discomfort in old patients and anxiety about the risk of cancer in relatively younger patients. These patients desire a minimally invasive procedure.

Transanal endoscopic microsurgery (TEM) was developed in 1983 by Büess *et al*[2-6]. It is a minimally invasive procedure to manage anorectal villous adenomas and early rectal adenocarcinomas[7,8]. TEM can be used to excise tumors located in the middle and upper rectum, and to manage other lesions, such as adenomas, which are not amenable to colonoscopic excision and can spare some patients the risks and side effects of major rectal surgery. In this study, patients had several large polyps and many small polyps. We performed TEM repeatedly to excise large polyps and fulgerize small polyps. The patients were satisfied with the clinical and anorectal functional outcome after TEM.

Generally, several factors from a single TEM procedure may cause internal anal sphincter (IAS) damage, which results in a decrease in mean anal resting pressure (ARP) and absence of rectoanal inhibitory reflexes (RAIR)[9]. It is unknown if repeated TEM will cause more severe IAS damage than a single TEM. The current study assessed anorectal function by anorectal manometry. Anorectal manometry provides objective data, which are necessary to determine sphincter function postoperatively.

MATERIALS AND METHODS

From January 2005 to June 2011, 21 patients (13 females and 8 males) who had been diagnosed with FAP and underwent TEM 2 or 3 times were included in the study. All the patients had undergone subtotal colectomy with ileorectal anastomosis previously. When there were more than 5 large (> 1 cm) polyps in the remaining rectum (range: 6-20 cm from the anal edge), including 19 villous adenomas and 2 adenocarcinomas (2 uT1N0M0), these patients underwent TEM at least twice (19 underwent TEM twice and 2 underwent TEM 3 times) in our department using the technique designed by Buess *et al*[2,10].

All these patients were followed up for more than 6 mo (range: 6-24 mo). The median age of these patients at the time of the operation was 66 years (range: 49-82 years). All of the 21 patients underwent colonoscopy, biopsy and anorectal manometry before operation, and 2 patients with malignant lesions underwent endosonography and magnetic resonance imaging for preoperative staging. The inclusion criteria for this study were: those patients who had documentation for TEM, with villous adenomas, and uT1N0M0 adenocarcinomas located 5-18 cm from the anal verge. The exclusion criteria were preoperative defects of IAS as shown by endosonography or preoperational anal incontinence.

For patients with benign or low-grade malignant polyps in the remaining rectum, we performed submucosal endoscopic excision with TEM. Intraoperative pathological examination was performed to ensure that no malignant lesion remained. We closed the defect with sutures for the large polyps (> 1 cm).

There are a variety of approaches for anorectal manometry and determining RAIR. In our study, anorectal manometry was performed with an 8-channel water-perfused catheter (MMS Corporation, Holland) with an external diameter of 5.5 mm. A computer system with menu-driven software (MMS Corporation, Holland) was used. The patients assumed the left lateral decubitus position. The stationary technique (the catheter was left in one position) was used in our laboratory. Anorectal manometric parameters, such as ARP, maximum squeeze pressure (MSP), maximum tolerable volume (MTV) and RAIR were recorded. Endoanal ultrasonography was used to study the integrity and thickness of the IAS and external anal sphincter (EAS). Manometry and a stan-

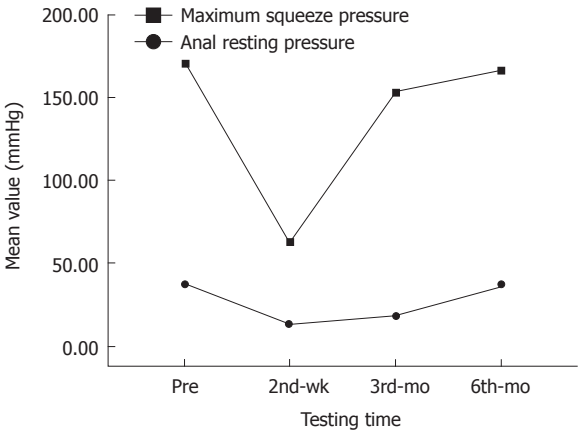


Figure 1 Changes in anal resting pressure and maximum squeeze pressure.

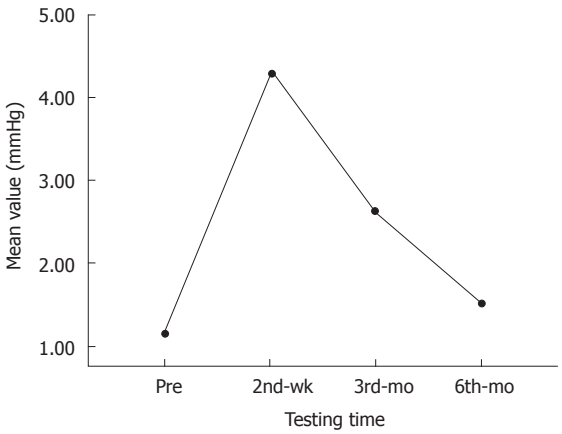


Figure 2 Changes in Wexner scores. The Wexner scores increased into a high level at postoperative week 2 ($P < 0.01$ *vs* preoperation). It returned to normal at postoperative month 6.

Table 1 Changes in mean anal resting pressure, maximal squeeze pressure and maximum tolerable volume ($n = 21$)

	Before operation	2nd wk after operation	3rd mo after operation	6th mo after operation
ARP (mmHg)	38 ± 5	14 ± 2[a]	19 ± 3[a]	37 ± 5
MSP (mmHg)	171 ± 19	62 ± 12[a]	166 ± 18	169 ± 20
MTV (mL)	165 ± 19	42 ± 14[a]	60 ± 11[a]	159 ± 19

[a]$P < 0.05$ *vs* perioperative values. ARP: Anal resting pressure; MSP: Maximal squeeze pressure; MTV: Maximum tolerable volume.

dardized questionnaire about incontinence were carried out at preoperative week 1 and postoperative week 2, months 3 and 6.

We used the SF-36 score, which summarizes physical and mental health status, to assess the effect of multiple TEM on patient quality of life (QoL).

SPSS 17.0 software was used for statistical analysis. Paired Student's *t* test was used to analyze the manometric data.

RESULTS

Apart from negative RAIR in 4 patients, all of the anorectal manometric values in the 21 patients were normal before the operation. However, all anorectal manometric parameters changed to different degrees after TEM. The mean ARP decreased from 38 mmHg ± 5 mmHg to 19 ± 3 mmHg (38 ± 5 mmHg *vs* 19 ± 3 mmHg, $P = 0.000$) and MTV decreased from 165 ± 19 mL to 60 ± 11 mL (165 ± 19 mL *vs* 60 ± 11 mL, $P = 0.000$) at month 3 after TEM. MSP decreased from 171 ± 19 mmHg to 62 ± 12 mmHg (171 ± 19 mmHg *vs* 62 ± 12 mmHg, $P = 0.000$) at week 2 after operation, and returned to normal values at postoperative month 3 (171 ± 19 *vs* 166 ± 18, $P = 0.051$). ARP and MTV were 37 ± 5 mmHg (38 ± 5 mmHg *vs* 37 ± 5 mmHg, $P = 0.057$) and 159 ± 19 mL (165 ± 19 mL *vs* 159 ± 19 mL, $P = 0.071$), respectively, at month 6 after TEM, which were up to the normal values (Figure 1). Among them, ARP in 18 patients, MSP in 19 patients and MTV in 21 patients were decreased by more than 50% at wk 2 after TEM (Table 1).

RAIR was absent in 4 patients preoperatively and in 12 ($\chi^2 = 4.947$, $P = 0.026$) patients at 3 mo after surgery while RAIR was absent only in 5 patients (including 4 patients whose RAIR was absent preoperatively) at postoperative month 6 ($\chi^2 = 0.141$, $P = 0.707$).

Endosonography demonstrated that disruption to the IAS occurred in 8 patients, with full integrity of the EAS in all patients. Of these 8 patients, 6 had incontinence to flatus at postoperative week 2, which disappeared at postoperative month 3. IAS thickness decreased from 1.9 ± 0.6 mm preoperatively to 1.3 ± 0.4 mm (1.9 ± 0.6 mm *vs* 1.3 ± 0.4 mm, $P = 0.000$) at postoperative month 3 and increased to 1.8 ± 0.5 mm (1.9 ± 0.6 mm *vs* 1.8 ± 0.5 mm, $P = 0.239$) at postoperative month 6. EAS thickness decreased from 3.7 ± 0.6 mm preoperatively to 3.5 ± 0.3 mm (3.7 ± 0.6 mm *vs* 3.5 ± 0.3 mm, $P = 0.510$) at month 3 and then increased 3.6± 0.4 mm (3.7 ± 0.6 mm *vs* 3.6 ± 0.4 mm, $P = 0.123$) at month 6, as demonstrated by endoanal ultrasonography.

Twenty patients had frequent stools per day and relatively high Wexner scores in a short time period after repeated TEM. Two-thirds of the patients had more than 5 bowel movements per day and 1 bowel movement at night. Bowel movements almost returned to the preoperative frequency in 17 of these patients 3-6 mo after surgery. Actual fecal incontinence was uncommon, but episodes of soiling were reported by 3 patients. Overall, the number of stools per day increased from 1.5 ± 0.7 before surgery to 5.6 ± 1.8 (1.5 ± 0.7 *vs* 5.6 ± 1.8, $P = 0.000$) at postoperative week 2 and then decreased to 1.7 ± 0.7 (1.5 ± 0.7 *vs* 1.7 ± 0.7, $P = 0.329$) at postoperative month 6. The Wexner scores increased from 0 before surgery to 4.1 ± 0.8 (0 *vs* 4.1 ± 0.8, $P = 0.000$) at postoperative week 2, and then they decreased to 2.0 ± 0.5 (0 *vs* 2.0 ± 0.5, $P = 0.002$) and 0.7 ± 0.1 (0 *vs* 0.7 ± 0.1, $P = 0.261$) at postoperative mos 3 and 6, respectively (Figure 2).

With regard to the QoL of the patients, the 2 scores of the SF-36, which summarizes the physical and mental health status, were 56.1 ± 6.1 and 46.2 ± 5.9, respectively (both are 50 in the general population). The scores were not significantly different compared with the general population.

All the patients were followed up for 6-24 mo, and no case of relapse was found.

DISCUSSION

FAP is a genetic disorder that results from a mutation in the adenomatous polyposis gene. In the majority of patients, polyps arise during childhood, mostly in the distal colon (rectosigmoid) as small intramucosal nodules. At adolescence, the polyps are usually identified throughout the colon and, thereafter, increase in size and number. Approximately half of FAP patients develop adenomas by 15 years of age and 95% develop by 35 years.

Given the substantial risk of rectal cancer developing after colectomy and ileorectal anastomosis, total proctocolectomy was recommended mostly for the typical FAP patients with multiple rectal adenomas. Currently, surgical options for FAP include subtotal colectomy with IRA, total proctocolectomy with ileostomy, and proctocolectomy with or without mucosectomy and ileal pouch anal anastomosis.

Therefore, TEM has gradually been accepted. TEM is a new technique used only in a few well-known hospitals in China, and excellent outcome in morbidity and a low relapse rate have been achieved. A few studies have addressed its functional results[11-17] and suggest that several factors during a single TEM may cause IAS damage. This damage results in a decrease in mean ARP and absence of RAIR[9]. There have been no studies on clinical and anorectal functional results after repeated TEM. It is unknown whether repeated TEM causes more severe IAS damage.

Several studies have shown that the IAS is the main contributor to ARP[18-20]. Division or damage of the IAS may be associated with troublesome soiling[21] and accounts for approximately 50%-85% of ARP, and EAS contributes to the other 25%-30% of ARP[22-24]. On the other hand, MSP is largely related to contraction of the EAS. Use of a wide rectoscope during TEM may result in rupture of the IAS because of significant anal dilatation, followed by a temporary decrease in ARP and MSP.

A fall in anorectal pressures because of anal dilatation can occur after using anal separators and stapling instruments in coloproctological procedures for hemorrhoids, fistula, or cancer[25-27]. According to our study results, the decrease in sphincteric thickness at postoperative month 3 due to anal dilatation may be another factor contributing to a temporary fall in anorectal pressure. Both ARP and MSP were decreased at postoperative week 2. However, MSP turned normal at postoperative month 3, which was faster than that of ARP and MTV at postoperative month 6. The integrity of both EAS and IAS could be explained by the finding that patients were continent, even though ARP and MSP were decreased in a short time period after surgery. Six of 8 patients with rupture of the IAS in our study presented with some degree of incontinence. We consider that a damaged IAS may result in temporary incontinence. MTV was decreased at postoperative week 2 and was returned to normal at postoperative month 6. Partial excision and reconstruction of the anorectal wall during TEM resulted in a decrease in anorectal volume and compliance, which may lead to a decrease in MTV.

RAIR is defined as transient relaxation of the IAS in response to rectal distension as confirmed by Denny-Brown and Robertson[28]. RAIR has been postulated as a factor in maintenance of continence[29,30], but the exact role of RAIR is unknown. Therefore, the upper anal canal is able to discriminate between flatus and fecal material. It is absent initially after low anterior resection and the ileoanal pouch procedure. Regeneration may occur after hand-sewn coloanal anastomosis and low stapled anastomosis[31-34]. RAIR disappears when the anorectal wall is removed[35] and when there are changes with depth if the IAS is excised[10]. According to our study results, RAIR was absent in 4 patients preoperatively and absent in only 5 patients (including the 4 patients whose RAIR was absent preoperatively) at postoperative month 6. Patients whose RAIR was absent after TEM had a deeper IAS excision. Only one patient (excluding the 4 patients whose RAIR was absent preoperatively) whose RAIR was absent postoperatively was treated with TEM 3 times and the excision of the anorectal wall was deeper.

We used the Wexner score to assess continence[36]. Most patients had more frequent stools per day (15 patients had more than 5 bowel movements per day and 1 bowel movement at night) and relatively higher Wexner scores (range: 6-13) at postoperative week 2 after multiple TEMs. There was remarkable improvement in these incontinence scores at 6 mo after operation, with most patients reporting satisfactory anorectal function. While actual fecal incontinence was unusual, episodes of soiling were reported by 3 patients.

With regard to the QoL of patients who had multiple TEM procedures, at 6 mo after operation, the physical and mental health status scores were not significant compared with the general population. The FAPs, multiple-operations, postoperative discomfort were possible explanations for the low mental health status scores. A good QoL is a surrogate for good functional outcome. In the current study, anorectal function was well-preserved, even though some anorectal manometric parameters changed over time.

In conclusion, we found that anorectal function is well-preserved when TEM is performed repeatedly and patients have a good QoL. Repeated TEM is a good choice of treatment for resection of multi-polyps in the remaining rectum of FAP patients who underwent subtotal colectomy.

COMMENTS

Background

Familial adenomatous polyposis (FAP) can cause colorectal cancer. Some of the patients with FAP who underwent subtotal colectomy in China twenty years ago refuse to undergo a complicated operation because of old age or poor physical status. The authors have used transanal endoscopic microsurgery (TEM) repeatedly to excise large polyps and fulgerize small polyps for preventing the canceration of the disease.

Research frontiers

TEM has been suggested as a minimally invasive procedure to manage ano-

rectal villous adenomas and early rectal adenocarcinomas. However, TEM procedure may cause internal anal sphincter (IAS) damage. It is unknown if several procedures of TEM cause more severe IAS damage than a single TEM. There had been no studies on clinical and anorectal functional outcome after repeated TEM.

Innovations and breakthroughs

It is unknown whether TEM that is performed multiple times causes more severe IAS damage. This is the first study to report that repeated TEM is a good choice of treatment for resection of multi-polyps in the remaining rectum of FAP patients who undergo subtotal colectomy. Furthermore, the study showed that a good anoretal functional outcome with little IAS damage was achieved after repeated TEM.

Applications

Repeated TEM is a good choice of treatment for resection of multi-polyps in the remaining rectum of FAP patients who underwent subtotal colectomy.

Peer review

This is a clinical report on anorectal function measured before and 2 to 3 after TEM excision of recurrent polyps in the rectal stump of FAP patients who underwent subtotal colectomy. This is a very interesting article which evaluates the short and long-term anorectal function (as measured by anorectal manometry) following repeat TEM procedures. TEM is very helpful tool in the management of recurrent polyps in FAP patients who have a residual rectal stump. There is little data on its use in this clinical setting, and no data on the impact of serial TEM procedures on anorectal function, so this work is important.

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CASE REPORT

Treatment of recurrent sigmoid volvulus in Parkinson's disease by percutaneous endoscopic colostomy

Susan Toebosch, Vera Tudyka, Ad Masclee, Ger Koek

Susan Toebosch, Department of Gastroenterology, Laurentius Hospital, 6043 CV Roermond, The Netherlands
Vera Tudyka, Department of Surgery, Laurentius Hospital, 6043 CV Roermond, The Netherlands
Ad Masclee, Ger Koek, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Maastricht University Medical Center, 6229 HX Maastricht, The Netherlands
Author contributions: Toebosch S wrote the paper, reviewed current literature, performed endoscopic treatment and clinical follow-up; Tudyka V wrote the paper and reviewed current literature; Masclee A revised the paper; Koek G performed endoscopic treatment and revised the paper.
Correspondence to: Susan Toebosch, MD, Consultant Gastroenterologist, Department of Gastroenterology, Laurentius Hospital, Monseigneur Driessenstraat 6, 6043 CV Roermond, The Netherlands. susantoebosch@hotmail.com
Telephone: +31-475-383028 Fax: +31-475-382525
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Abstract

The exact aetiology of sigmoid volvulus in Parkinson's disease (PD) remains unclear. A multiplicity of factors may give rise to decreased gastrointestinal function in PD patients. Early recognition and treatment of constipation in PD patients may alter complications like sigmoid volvulus. Treatment of sigmoid volvulus in PD patients does not differ from other patients and involves endoscopic detorsion. If feasible, secondary sigmoidal resection should be performed. However, if the expected surgical morbidity and mortality is unacceptably high or if the patient refuses surgery, percutaneous endoscopic colostomy (PEC) should be considered. We describe an elderly PD patient who presented with sigmoid volvulus. She was treated conservatively with endoscopic detorsion. Surgery was consistently refused by the patient. After recurrence of the sigmoid volvulus a PEC was placed.

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Key words: Colonic dysfunction; Colostomy; Endoscopic treatment; Parkinson's disease; Sigmoid volvulus

Peer reviewer: Dr. Ali Harlak, Department of General Surgery, Gulhane Military Medical Academy, GATA Genel Cerrahi AD, 06018 Ankara, Turkey

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INTRODUCTION

Gastrointestinal symptoms are common in Parkinson's disease (PD) and may give rise to life threatening conditions such as sigmoid volvulus. Early recognition and treatment of constipation in PD patients may alter complications such as sigmoid volvulus. We describe a patient known to have PD with recurrent bowel dilatation associated with sigmoid volvulus. Furthermore, we review the relevant literature concerning volvulus in patients with PD and treatment of this condition.

CASE REPORT

A 73-year-old female patient was admitted three times within three months to the gastrointestinal ward with recurrent painless abdominal distension. A week before her first admission she noticed progressive abdominal distension. Apart from nausea she experienced no other symptoms.

Her past medical history revealed breast carcinoma for which she had undergone a modified radical mastectomy in 1997 and resection of local recurrence combined with hormonal therapy in 2005. She was also known to have severe PD for more then twenty years.



Figure 1 Typical "bean-shaped" sigmoid with distension up to 11 cm representing sigmoid volvulus.



Figure 3 Colonic dilatation resolved after endoscopic detorsion with large bore rectal tube placement.

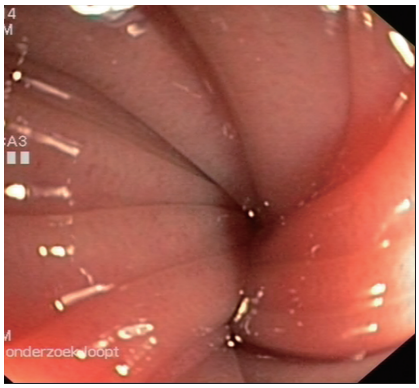


Figure 2 Endoscopic image of torsion of sigmoid: "Toffee-sign".

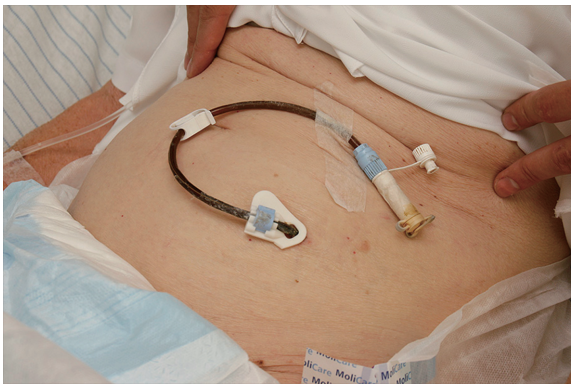


Figure 4 Percutaneous endoscopic colostomy has been placed.

Medication consisted of levodopa/carbidopa and amantadine.

On physical examination at admission the patient was not very ill and had normal vital parameters. Her abdomen was grossly distended and high pitched bowel sounds were heard. The clinical diagnosis of an ileus was made. Laboratory tests were unremarkable without any sign of inflammation. An abdominal X-ray revealed a typical coffee bean-shaped sigmoid with gross dilatation up to 11 cm (Figure 1).

The patient was treated with enemas and laxatives. A colonoscopy was performed which did not reveal any mucosal irregularities. At 40 cm from the anus a strangulation of the sigmoid was seen with proximal dilatation of the colon which is pathognomonic for volvulus (Figure 2). Surgical treatment was repeatedly considered but consistently refused by the patient. Decompression of the colon was finally established by a large (11 French) cannula inserted proximal from the evident torsion of the sigmoid (Figure 3). Ten days after admission the patient was discharged in good clinical condition.

However, two months later she was readmitted with a recurrence of sigmoid volvulus. For the second time, conservative treatment with endoscopic derotation of the affected sigmoid and placement of a cannula for decompression was performed. To prevent further recurrence, a percutaneous endoscopic colostomy (PEC) was placed in the distal colon to achieve fixation to the abdominal wall (Figure 4). No complications occurred. The patient was discharged a few days later. Two years later she was admitted with a third recurrence of sigmoid volvulus and was again successfully treated with endoscopic derotation. The patient still persists in her wish not to be operated on.

DISCUSSION

Colonic dysfunction in PD

Parkinsonism is an extrapyramidal syndrome with symptoms consisting of a variable combination of general rigidity, disturbance of posture, gait and tremor. The signs of this chronic and progressive disease, affecting mostly people of sixty-five years and older, are caused by loss of nerve cells in the pigmented substantia nigra pars compacta and the locus coeruleus in the midbrain. Apart from typical parkinsonian features as mentioned above, gastrointestinal symptoms are common, especially motility disorders, varying from swallowing disorders, gastroparesis to bowel dysmotility and anorectal dysfunction[1-3]. Constipation is one of the most common gastrointestinal features in PD patients. Constipation may even precede typical symptoms 10 or 20 years before the onset of PD[4-7]. Several reports have revealed that constipation occurs in up to 50% of patients with PD. The severity of constipation seems be directly related to the severity and duration of PD[1].

There is a multiplicity of factors contributing to constipation in PD patients. Reduced physical activity and a decrease in swallowing saliva may play a role in constipation. Antiparkinsonian drugs are thought to contribute

to diminished bowel motions[8], although an association between constipation and PD was noted even before the introduction of antiparkinsonian drugs by Parkinson in 1817. Several studies did not confirm the correlation between medication and delayed colonic transit time[1,9]. In PD, colonic transit time is prolonged in all segments of the colon[8-11]. Defecation itself can be impaired by decreased phasic rectal contractions, weak abdominal strain and/or paradoxical sphincteric contractions during defecation[10]. However, the exact pathophysiology of autonomic gut dysfunction remains obscure.

Constipation may result from both peripheral as well as central nerves. Depletion of dopamine-containing neurons in the central nervous system is a basic defect in PD. On the other hand, a substantial decrease in dopaminergic myenteric neurons has been documented[12]. Deposition of intracytoplasmic hyaline inclusions, so-called Lewy bodies, can be found not only centrally in the pigmented nuclei but also in the myenteric and submucosal plexus[13]. Conditions associated with constipation in PD patients include megacolon and sigmoid volvulus[14,15].

Volvulus and PD

In sigmoid volvulus there is twisting of the sigmoid around its mesenteric axis. Apart from obstruction it may also involve strangulation of the main vessels at the base of the affected mesentery. Sigmoid volvulus is the third leading cause of large bowel obstruction in adults and is even more common in the elderly[16-18]. It can be a life threatening condition with high mortality rates of up to 50%[19].

The incidence of sigmoid volvulus in PD is not known. Only a few reports have been published concerning this association[14,20-22]. A longstanding history of constipation is, amongst anatomic variations and neuropsychiatric diseases, one of the risk factors for the development of volvulus because of an abnormally loaded loop of bowel[23,24]. The diagnosis of volvulus is based on physical examination in combination with an abdominal X-ray revealing a typical "coffee bean" shaped sigmoid. In the majority of cases no further investigation is required to confirm the diagnosis of volvulus[25]. If there is any doubt about possible obstructive disease, an abdominal computed tomography-scan can be considered. However, colonoscopy is the most favourable diagnostic tool to visualize obstructive colonic moments. The endoscopic picture of volvulus shows a pinpoint lumen with wrenched sides. When passing the pinpoint opening one sees a grossly dilated lumen (Figure 2). In the case of volvulus, endoscopy allows immediate treatment.

Management of sigmoid volvulus

Awareness of the high incidence of gastrointestinal disorders, and especially constipation, in PD patients is mandatory in order to recognise a condition such as sigmoid volvulus. The question remains as to whether the aetiology of sigmoid volvulus in this specific group of patients is different. If constipation is the main cause, the primary focus should be on medical prophylactic treatment with laxatives. However, if patients with PD have redundant sigmoid, surgery remains the first choice of treatment. No data have been published related to this question. Even for volvulus in general, the published data are too scarce to allow high level evidence-based judgement.

Early aggressive treatment of constipation with dietary adjustments, physiotherapy, bulk-forming agents and laxatives should be the aim in all PD patients. The prokinetic agent, cisapride, has been shown to reduce colonic transit time in PD patients, but seemed to be ineffective after long-term use[9] and is no longer regularly available. Tegaserod, a 5-hydroxytryptamine type 4 agonist, has shown to improve bowel movement frequency and stool consistency[26]. In addition, improvement in subjective experience of symptoms by PD patients has been observed[27]. Further trials are not expected since this drug was also withdrawn because of an increased risk of serious cardiovascular adverse events.

In patients with alarming signs of possible necrosis or perforation, immediate surgical intervention is needed. When symptoms are mild and not worrisome, urgent endoscopic detorsion should be considered as the first choice of treatment[16,25,28]. Endoscopy enables direct assessment of the viability of the colonic mucosa. Although endoscopic detorsion has high recurrence rates, varying from 3.3% to over 60%[29,30] , it is a relatively safe procedure with mortality rates of 1%-3%[29,31].

In cases of successful endoscopic detorsion, semi-elective sigmoidectomy can be performed. This allows an appropriate workup to achieve optimal preoperative conditions. Mortality rates vary between 0% and 10%[16,23,32,33].

In recurrent volvulus, surgery is the first choice of treatment as endoscopy in recurrent volvulus is accompanied by a raised mortality rate of up to 20%[16]. However, surgical treatment in the acute phase of volvulus is also known to have high mortality[16,17,23,29,31].

There are several surgical options to treat volvulus, ranging from operative detorsion alone to retroperitoneal fixation and partial resection with or without primary anastomosis. Detorsion alone has a very high recurrence rate of up to 74%[16,31].

Resection performed with primary anastomosis is advocated by some authors as it has the advantage of avoiding a second operation to restore continuity[25,28,32]. Mortality in this group ranges from 0%-19%[23,28,33]. If the expected surgical morbidity and mortality is unacceptably high or if patients refuse surgery, as in our case, PEC can be considered. Under endoscopic vision, a tube is placed percutaneously in the distal colon. This allows fixation of the colon to the abdominal wall and if necessary offers direct access for laxatives and desufflation. Complications following PEC such as infection, faecal leakage and buried internal bolster can cause significant

morbidity, but is a good alternative in selected cases[34,35]. There are few available publications on recurrence rate after PEC[34,35].

In conclusion, gastrointestinal dysfunction is a common feature in elderly patients, and especially in those affected by PD. Patients with chronic constipation are more likely to develop a complication like sigmoid volvulus. The association between sigmoid volvulus and PD has been described previously. There are no data available suggesting different treatment for sigmoid volvulus in PD patients. In general, endoscopic detorsion can be performed as the first choice of treatment in acute sigmoid volvulus if signs of an acute abdomen are absent. This should be followed by resection at a later stage if the condition of the patient allows surgical intervention. However, if there are signs of gangrene, an emergency operation is the only therapeutic option and endoscopic treatment should not delay surgery. PEC is an alternative treatment for selected cases if surgical intervention is not an option.

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CASE REPORT

Sarcoidosis and chronic hepatitis C: A case report

Vadim Brjalin, Riina Salupere, Valentina Tefanova, Kaiu Prikk, Natalia Lapidus, Enn Jõeste

Vadim Brjalin, Department of Internal Medicine, West-Tallinn Central Hospital, 10617 Tallinn, Estonia
Vadim Brjalin, Riina Salupere, Department of Internal Medicine, University of Tartu, 51014 Tartu, Estonia
Valentina Tefanova, Department of Virology, National Institute for Health Development, 11619 Tallinn, Estonia
Kaiu Prikk, Institute of Clinical Medicine, Tallinn University of Technology, 19086 Tallinn, Estonia
Natalia Lapidus, Department of Pathology, West-Tallinn Central Hospital, 10617 Tallinn, Estonia
Enn Jõeste, Department of Pathology, North Estonia Medical Centre, 13419 Tallinn, Estonia
Author contributions: All authors participated equally in the preparation of this case report by contributing their expertise; Brjalin V and Tefanova V wrote the text of the manuscript; Salupere R reviewed the manuscript and made final improvements to the manuscript; Prikk K managed the patient with sarcoidosis; Lapidus N and Jõeste E examined the histological specimens; all authors approved the final manuscript.
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Correspondence to: Vadim Brjalin, MD, Department of Internal Medicine, West-Tallinn Central Hospital, Paldiski Road 68, 10617 Tallinn, Estonia. vbrjalin@gmail.com
Telephone: +372-65-11472 Fax: +372-65-11472
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Abstract

Several case reports deal with the relationship between hepatitis C virus (HCV) infection and pulmonary or hepatic sarcoidosis. Most publications describe interferon α-induced sarcoidosis. However, HCV infection *per se* is also suggested to cause sarcoidosis. The present case report describes a case of biopsy-verified lung and liver sarcoidosis and HCV infection, and the outcome of antiviral therapy. In March 2009, a 25-year-old man presented with moderately elevated liver enzymes without any clinical symptoms. The patient was positive for HCV antibodies and HCV RNA of genotype 1b. Four months later the patient became dyspnoic and pulmonary sarcoidosis was diagnosed by lung biopsy and radiography. A short course of corticosteroid treatment relieved symptoms. Three months later, liver biopsy showed noncaseating granulomas consisting of epithelioid histiocytes and giant cells with a small amount of peripheral lymphocyte infiltration, without any signs of fibrosis. Chronic HCV infection with coexistence of pulmonary and hepatic sarcoidosis was diagnosed. Antiviral therapy with peginterferon α and ribavirin at standard doses was started, which lasted 48 wk, and sustained viral response was achieved. A second liver biopsy showed disappearance of granulomas and chest radiography revealed normalization of mediastinal and perihilar glands. The hypothesis that HCV infection *per se* may have triggered systemic sarcoidosis was proposed. Successful treatment of HCV infection led to continuous remission of pulmonary and hepatic sarcoidosis. Further studies are required to understand the relationship between systemic sarcoidosis and HCV infection.

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Key words: Pulmonary and hepatic sarcoidosis; Hepatitis C virus infection; Sustained viral response; Peginterferon α; Ribavirin

Peer reviewers: Dr. Mihaela Petrova, MD, PhD, Clinic of Gastroenterology, Medical Institute, Ministry of Interior, 1606 Sofia, Bulgaria; Dr. Justin MM Cates, MD, PhD, Department of Pathology, Vanderbilt University Medical Center, Medical Center North, C-3322, 1161 21st Avenue South, Nashville, TN 37232, United States

Brjalin V, Salupere R, Tefanova V, Prikk K, Lapidus N, Jõeste E. Sarcoidosis and chronic hepatitis C: A case report. *World J Gastroenterol* 2012; 18(40): 5816-5820 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i40/5816.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i40.5816

INTRODUCTION

The estimated global prevalence of hepatitis C virus (HCV) infection is around 3%, corresponding to 170

million infected people[1]. As estimated, up to 1% of 1.34 million of the Estonian population are infected with HCV and this virus was reported to be the main etiological agent for chronic hepatitis and liver cirrhosis in Estonian patients[2]. After acute infection, chronic hepatitis develops in up to 80% of cases and may lead to hepatic and extrahepatic complications. There have been reported more than 30 extrahepatic manifestations of chronic HCV infection[3]. The best documented manifestation is essential mixed cryoglobulinemia. Non-cryoglobulin diseases having a relationship with HCV include idiopathic pulmonary fibrosis, lichen planus, Sjogren syndrome, autoimmune thyroiditis, porphyria cutanea tarda, chronic polyarthritis and some others[4].

Sarcoidosis is a systemic inflammatory disease of unknown etiology with the presence of noncaseating epithelioid cell granulomas in many organs. The diagnosis of sarcoidosis is based on a compatible clinical and/or radiological picture, histological evidence of noncaseating granulomas and the exclusion of other diseases having a similar histological or clinical picture[5].

The prevalence of sarcoidosis in the general population varies throughout the world from less than one case to 40 cases per 100 000 people or 0.001%-0.04%[5,6]. The prevalence of sarcoidosis in HCV-infected patients is reported to be 0.1%-0.2%[7,8]. A relationship between systemic sarcoidosis and HCV infection may emerge due to antiviral therapy with interferon α and ribavirin (in 75% of cases), or owing to unknown factor(s)[9]. Coexistence of sarcoidosis and untreated HCV infection prompted some authors to suggest that HCV infection *per se* can induce sarcoidosis[10]. The decision to start antiviral therapy for HCV infection in patients with pre-existing sarcoidosis is a delicate challenge for the clinician, calling for close monitoring[9].

This case report describes the clinical and histological characteristics of systemic sarcoidosis and chronic HCV infection, and the successful outcome of antiviral therapy.

CASE REPORT

In March 2009, a 25-year-old male patient was referred to the gastroenterologist at the West-Tallinn Central Hospital with moderately elevated liver enzymes: alanine aminotransferase (ALAT) 111 U/L (reference range < 42 U/L), aspartate aminotransferase 44 U/L (reference range < 37 U/L); and with positive HCV antibodies. According to his case history, elevated liver enzymes were also observed during a routine visit to the family doctor in December 2008.

The patient denied intravenous drug abuse, any occupational exposures and blood transfusions in the past. The time and manner of acquisition of HCV infection remained uknown. The patient did not have any symptoms of liver disease. Human immunodeficiency virus, surface antigen of the hepatitis B virus and autoantibodies; i.e., antinuclear antibodies, anti-mitochondrial M2-antibodies, anti-liver-kidney microsome antibodies and anti-smooth muscle antibodies, were negative. The values of ferritin, thyroid-stimulating hormone and ceruloplasmin were within the reference ranges. The patient was infected with HCV genotype 1b determined by the hybridization technique (VERSANT HCV genotype assay); the viral load was 723 000 IU/mL as analysed by the polymerase chain reaction assay (COBAS® AmpliPrep/COBAS® TaqMan). Abdominal ultrasound revealed no changes in liver size and appearance, nor was there noted any pathology in the other internal organs.

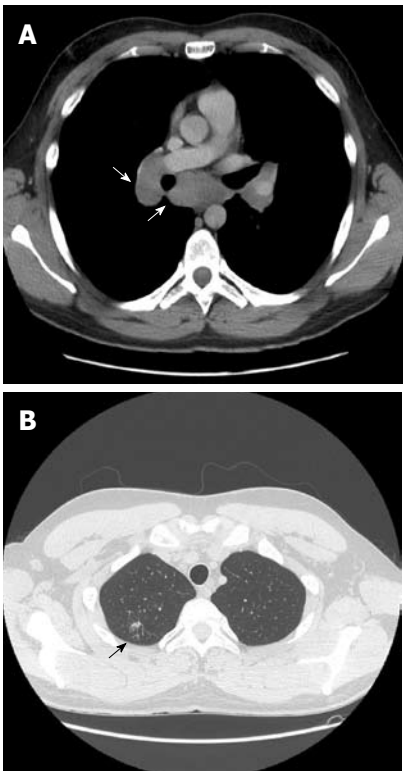


Figure 1 Thoracic computer tomographic scans of mediastinal, hilar lymph nodes and pulmonary nodules. Enlarged lymph nodes (A, white arrows) and small pulmonary nodules in the right upper lobe before prednisolone treatment (B, black arrow; July 2009) are revealed.

The diagnosis was chronic hepatitis C of unknown origin. Antiviral treatment was offered, but the patient for some reason postponed treatment and decided to start it several months later.

In July 2009, the patient was referred to the pulmonologist due to erythema nodosum on his legs, and dyspnea and cough. Computer tomography (CT) scans of the chest showed mediastinal and hilar adenopathy and focal lesions in the right upper lung lobe (Figure 1). Transbronchial biopsy of pulmonary lymph nodes was performed, which revealed epithelioid cell granulomas (Figure 2). Systemic sarcoidosis was diagnosed and corticosteroid treatment with prednisolone 20 mg orally was started. After one month of corticosteroid therapy the skin lesions disappeared. As the patient did not experience any symptoms, he discontinued prednisolone treatment.

In October 2009, the patient still had elevated ALAT (60 U/L) and the viral load was 687 000 IU/mL. Ultra-

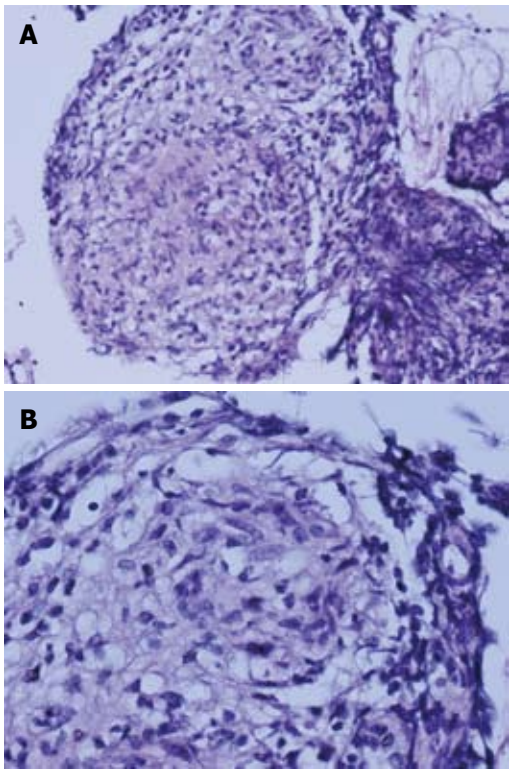


Figure 2 Biopsy taken from a pulmonary lymph node. Two tight naked round well-formed granulomas are surrounded by a small amount of lymphocytic infiltration. The granulomas consist of epithelioid cells; there are no necrosis, giant cells, Shaumann or asteroid bodies. Stained with hematoxylin and eosin, magnification × 200 (A) and × 400 (B).

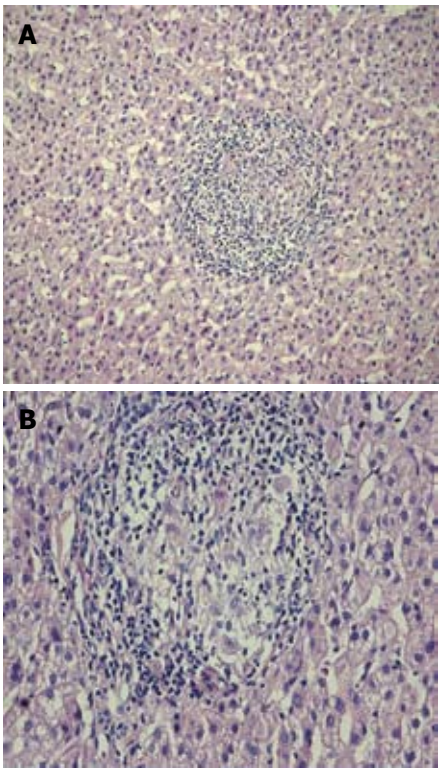


Figure 3 Liver biopsy revealing a noncaseating granuloma consisting of epithelioid histiocytes and giant cells with a small amount of peripheral lymphocyte infiltration. Stained with hematoxylin and eosin, magnification × 200 (A) and × 400 (B).

sound-guided liver biopsy was performed. It revealed 3 noncaseating granulomas consisting of epithelioid histiocytes and giant cells with weak peripheral lymphocyte infiltration, without any signs of fibrosis specific for chronic hepatitis C (Figure 3). His chest CT scans showed mild regression of lung sarcoidosis: mediastinal lymph nodes had became smaller while all other pathological findings remained the same (Figure 4A and B). The patient was diagnosed with chronic hepatitis C with coexisting pulmonary and hepatic sarcoidosis.

Taking into consideration that there was no exacerbation of pulmonary sarcoidosis and that the patient had chronic hepatitis C, antiviral therapy was opted for: peginterferon α-2a (Pegasys, F.Hoffmann La Roche Ltd, Basel, Switzerland) at a dose of 180 μg once a week subcutaneously and 1200 mg of ribavirin (Copegus, F.Hoffmann La Roche Ltd, Basel, Switzerland) daily orally was started. At week 4, the viral load decreased to 4755 IU/mL and no HCV RNA was found at weeks 12 and 24. The patient completed antiviral therapy at week 48 with normal liver enzymes and negative HCV RNA. At the end of treatment, chest CT scans showed a decrease in mediastinal and hilar adenopathy with disappearance of focal lesions in the upper lobe of the right lung (Figure 4C and D).

During treatment the patient experienced some side effects (fatigue, weight loss of 5 kg); he developed neutropenia and anemia but there was no need to change the doses of antiviral medications.

Six months after the end of treatment the patient had negative HCV RNA and his liver enzymes were normal. Sustained viral response (SVR) was documented. CT scans showed small mediastinal lymph nodes about 0.5 cm in diameter, and absence of hilar adenopathy and focal lesions in the upper lobe of the right lung.

Five months after SVR was confirmed, a new liver biopsy was performed which showed no remnant granulomas and mild steatosis in the liver tissue (Figure 5). No corticosteroid treatment was given after the first treatment course in July.

DISCUSSION

Sarcoidosis is a chronic multi-systemic granulomatous disease of unknown etiology related to exaggerated cellular immunological reactions[11]. The lungs, the liver, the lymph nodes, and the skin are commonly involved.

Here, we present a case of systemic sarcoidosis with the example of a treatment-naive patient with HCV 1b infection. The patient was first diagnosed as symptom-free with chronic HCV infection. Four months later, lung symptoms developed and the diagnosis was pulmonary sarcoidosis. The complaints - cough and dyspnea, as well as erythema nodosum - were relieved by the administration of oral corticosteroids. Three months later a liver biopsy revealed hepatic granulomas. Chronic HCV infection with coexistence of pulmonary and he-

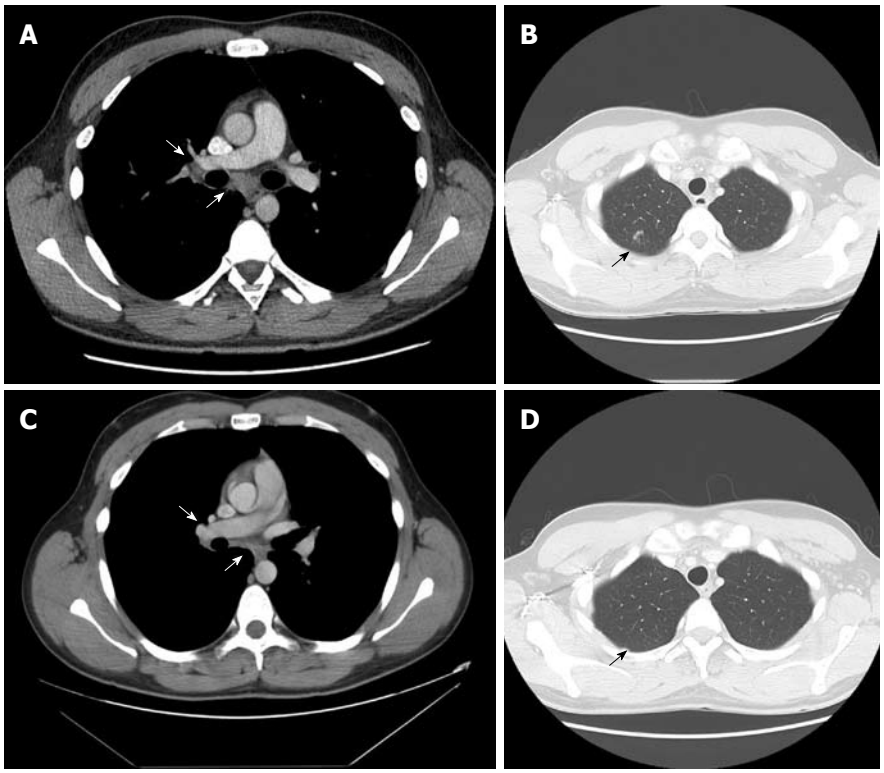


Figure 4 Thoracic computer tomographic scans of mediastinal, hilar lymph nodes and pulmonary nodules. A significant decrease in size of mediastinal and hilar lymph nodes (A, white arrows) is shown before initiation of antiviral treatment but pulmonary nodules remain at the same levels (B, black arrow; October 2009). At the end of treatment no progression of lymph nodes is revealed (C, white arrows) and pulmonary nodules are absorbed (D, black arrow; December 2010).

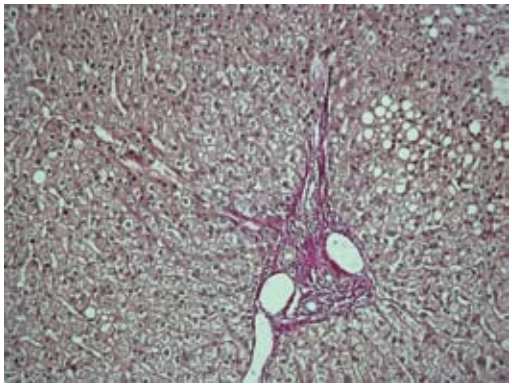


Figure 5 Liver biopsy revealing the absence of granuloma formation with vesicles of steatosis. Stained with van Geason, magnification × 200.

patic sarcoidosis was diagnosed. The patient started and completed a full 48-wk course of antiviral therapy without aggravating lung symptoms.

According to Ramos-Casals *et al*[9], sarcoidosis during antiviral therapy (in 75% of cases) has mainly a benign uncomplicated evolution, while more complicated cases are observed especially in HCV patients with preexisting sarcoidosis both with and without previous antiviral therapy.

In our studied HCV treatment-naive patient, systemic sarcoidosis was unrelated to antiviral therapy. In sarcoidosis observed in treatment-naive patients with HCV infection, skin involvement is less frequent than in sarcoidosis triggered by antiviral therapy. In the former case, systemic corticosteroids have to be used more often and the outcome appears less favorable[8]. In our patient, corticosteroid therapy for sarcoidosis was given for only a month when the patient discontinued therapy. Despite this, the outcome of therapy was favorable: the skin lesions disappeared, and progression of lung sarcoidosis or exacerbation of HCV infection was not noted.

In the case of pre-existing sarcoidosis the decision about starting antiviral therapy under close monitoring should be made very cautiously[8,9]. According to Charlier *et al*[12], reactivation of pre-existing sarcoidosis in HCV patients is rare and the manifestations of the disease are not very severe. Bearing these aspects in mind, antiviral therapy with peginterferon α and ribavirin for treatment of chronic HCV infection was opted for in the present case. The therapy was successful and the patient achieved SVR. No reactivation of systemic sarcoidosis with skin, lung or hepatic involvement was observed. The absence of hepatic granuloma on the second liver biopsy, performed five months after SVR, demonstrated a favorable treatment result possibly preventing hepatic granuloma in our patient, which develops into liver cirrhosis with life-threatening variceal bleeding in about 1% of these cases[13].

Our case report, as it considers hepatic involvement, is interesting in several aspects. Firstly, for sarcoidosis in the liver, the course of which is usually mild and tran-

sient, and clinically silent, granulomatous lesions are usually very small and asymptomatic[14,15]. In our patient the manifestations of liver disease were asymptomatic.

Corticosteroid treatment of hepatic sarcoidosis is used only for patients with severe liver lesions, such as chronic cholestatic disease, portal hypertension and cirrhosis[16].

Secondly, hepatic granulomas are not very rare in HCV disease. The prevalence of hepatic granulomas in liver biopsies of patients with chronic hepatitis C have been reported to be from 0.8% up to 9.5%[17-19] and only when other etiological factors of granuloma formation are excluded may it be considered as a part of the histologic spectrum of HCV[19]. In our treatment-naive HCV patient, noncaseating granuloma was found first in the lung lymph node and later liver biopsy also revealed granulomas, so coexistence of systemic sarcoidosis and chronic hepatitis C was diagnosed.

All these facts show that some similar specific immune pathogenic mechanisms may be involved in the course of systemic sarcoidosis and chronic HCV. It is possible that complex dysregulation of the cytokine/chemokine network in patients with HCV infection along with some other host factors might induce the release of certain mediators and activation of mononuclear phagocytes that finally cause a granulomatous reaction[20]. Although coexistence of both diseases in our case may be a coincidence, we, like Bonnet *et al*[10], suggest that HCV *per se* could be an antigenic trigger for development of sarcoidosis.

As systemic sarcoidosis may be reactivated after antiviral treatment, further close follow-up of our patient is needed.

In conclusion, physicians should be aware that HCV-infected patients may have systemic sarcoidosis unrelated to antiviral treatment. Also, it is obvious that further case-control studies focusing on sarcoidosis in patients with HCV infection and the relationship between sarcoidosis and antiviral therapy are needed.

CONSENT

Written informed consent was obtained from the patient for publication of this case report and accompanying images.

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CASE REPORT

Different imaging findings of inflammatory myofibroblastic tumor of the liver

Xiao-Fei Liu, Bao-Ming He, Xiao-Hui Ou-Yang, Zhi-Zhong Wang, Jia-Gui Su

Xiao-Fei Liu, Bao-Ming He, Xiao-Hui Ou-Yang, Zhi-Zhong Wang, Jia-Gui Su, Department of Nuclear Medicine, The People's Liberation Army 309th Hospital, Beijing 100091, China
Author contributions: Liu XF and He BM designed the research; Ou-Yang XH, Wang ZZ and Su JG contributed analytical tools; Liu XF and He BM wrote the paper.
Correspondence to: Bao-Ming He, MD, Department of Nuclear Medicine, The People's Liberation Army 309th Hospital, Beijing 100091, China. liufly301@163.com
Telephone: +86-10-51520604 Fax: +86-10-51520931
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Abstract

Inflammatory myofibroblastic tumor (IMT) in the liver is an uncommon lesion of uncertain pathogenesis. In most cases, symptomatological imaging and clinical studies suggest malignancy. We report a case of liver IMT with imaging findings from positron emission tomography/computed tomography (PET/CT), contrast-enhanced computed tomography (CECT) and contrast-enhanced ultrasonography (CEUS). This report was the first to depict a PET/CT scan of a liver IMT that revealed an inhomogeneous, intense (fluorine 18)-fluoro-2-deoxy-D-glucose uptake. The CECT and CEUS images showed a hepatic artery supplying blood to the mass and necrosis. The characteristic histopathological features and the presence of spindle cells expressing smooth muscle actin, collagen fibers and lymphocytes allowed for the diagnosis of liver IMT. Recognizing such findings will help to achieve a correct diagnosis and may prevent inappropriate treatment.

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Key words: Inflammatory myofibroblastic tumor; Liver; Emission-computed tomography; X-ray-computed tomography

Peer reviewer: Ming-Lung Yu, MD, PhD, Professor, Division of Hepatology, Department of Medicine, Kaohsiung Medical University Hospital, 100 Tzyou 1st Rd, Kaohsiung 807, Taiwan, China

Liu XF, He BM, Ou-Yang XH, Wang ZZ, Su JG. Different imaging findings of inflammatory myofibroblastic tumor of the liver. *World J Gastroenterol* 2012; 18(40): 5821-5825 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i40/5821.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i40.5821

INTRODUCTION

Inflammatory myofibroblastic tumor (IMT) is rare. Although IMT was once considered a soft tissue lesion, it has also been reported in the mesentery, omentum, major salivary glands, larynx, breast and thyroid gland, and in visceral organs, such as the lung, small intestine and kidney. To date, the appearance of IMT in the liver has been minimal[1]. Because of how it appears on different types of imaging, IMT is difficult to clinically differentiate from malignant tumors. IMT may regress spontaneously; therefore, indications for surgery must be carefully assessed. Cases involving (fluorine 18)-fluoro-2-deoxy-D-glucose positron emission tomography and computed tomography (^{18}F-FDG-PET/CT) examinations of liver IMT have never been reported. Here, we report a case of liver IMT using ^{18}F-FDG-PET/CT, contrast-enhanced computed tomography (CECT) and contrast-enhanced ultrasonography (CEUS). The imaging findings showed a hepatic artery supplying blood to the mass in the presence of necrosis. An inhomogeneous, intense ^{18}F-FDG uptake was also noted. The characteristic histopathological features, along with the presence of spindle cells expressing smooth muscle actin (SMA), collagen fibers and lymphocytes, allowed for the IMT diagnosis. Recognizing such findings will improve correct diagnosis and may prevent inappropriate treatment.

CASE REPORT

A 42-year-old Chinese woman presented with high fever (39 ℃) and denied having abdominal pain, fatigue and weight loss. On physical examination, the right upper quadrant of the patient's liver was tender at palpation. After being placed on anti-infection medicine, her body temperature decreased to a maintainable range (within normal limits). However, when the treatment ceased, the original fever returned. The laboratory results revealed a white blood cell count of 10 900/mL with segmental neutrophilia (73.4%) and slightly elevated levels of C-reactive protein (CRP, 116.2 mg/L) and fibrinogen (5.191 g/L); the erythrocyte sedimentation rate (ESR) and hemoglobin level (7.4 g/dL) were normal. The liver function tests were within the normal limits, as were the tests for alpha-fetoprotein (AFP, 2.0 ng/mL), carbohydrate antigen 19-9 (CA19-9, 13.84 U/mL), CA125 (17.71 U/mL), CA50 (12.4 U/mL) and carcinoembryonic antigen (CEA, 1.91 ng/mL). The serology markers for hepatitis and infectious agents such as echinococcus were also negative.

Conventional ultrasonography (CUS, ProSound SSD Alpha 10 system designed by Aloka Co, Ltd, Tokyo, Japan) revealed that there was a 3.8 cm × 4.6 cm hypoechoic mass in the right anterior lobe of the liver that had a clearly defined border and a heterogeneous echo texture. CEUS examinations were performed after the CUS by administering a sulfur hexafluoride-filled microbubble contrast agent (SonoVue; Bracco SpA, Milan, Italy). After administering the contrast agent, the arterial phase images showed hyperenhancement of the mass. The portal phase and parenchymal phase images revealed wash-out that corresponded to that on the arterial phase images. On the edge of the mass, there was a 1.3 cm × 1.5 cm solid nodule with a strong echo and a clear border that did not show signs of either parenchymal or arterial phases. CEUS indicated that the mass had a rich blood supply and necrosis, which is typical of a malignant tumor (Figure 1).

The patient had a whole-body ^{18}F-FDG PET/CT (Discovery VCT, GE, American) scan that returned normal results for all of the organs except the liver. The imaging showed an inhomogeneous, intense uptake of ^{18}F-FDG with SUVmax 9.1 (SUV standardized uptake value) in the anterior segment of the right lobe of the liver (Figure 2). The inhomogeneous uptake of the mass was more intense at its periphery. The mass size was approximately 4.9 cm × 4.6 cm × 5.0 cm. An abdominal CECT confirmed tumor localization in the anterior segment of the right lobe of the liver and showed that enhancement of the mass was broad and well defined at its periphery in the arterial phase. Portal venous phase images depicted a further enhancing mass, while the delayed-phase images revealed a quick wash-out that corresponded to the observations from the arterial-phase images. They were not as prevalent as those reported during the portal venous phase. There was evidence of necrosis in front of the mass and a liver lobe artery at the periphery of the mass. A 1.6 cm × 1.5 cm low-density nodule that had not been illuminated during either of the phases was present on the front edge of the mass (Figure 3).

Laparoscopy indicated that the mass was protruding from the liver surface and was slightly adhered to the surrounding tissue. The mass color was yellowish; however, it did not cause congestion or edema, and the common bile duct was not enlarged. The gall bladder, stomach, spleen, colon, small intestine and other organs were normal. A right lobectomy of the liver was performed by laparoscopy, and the mass was completely resected because the minimal contact it had with the large vessels minimized the risk. A portion of the free necrotic liver tissue was removed. Doctors dissected the specimen, which was approximately 6 cm in diameter.

Upon closer pathological examination, the mass measured 4.5 cm × 4.3 cm × 3.5 cm and weighed 1.6 kg; it was easily removed from the surrounding parenchyma. A microscopic examination revealed that the mass was composed of collagen fibers, chronic inflammatory cells lying on loose T-lymphocytes and ganglion-like cells. The chronic inflammatory infiltrate was characterized by the predominant proliferation of plasma cells in addition to lymphocytes and collagen fibers. An immunohistochemical analysis revealed that the spindle cells were positive for SMA and that the ganglion cell-like cells were positive for neuron-specific enolase and S-100. Staining for P53, desmin and epithelial membrane antigen was negative. The histological patterns were fibrous histiocytoma-like and desmoid-type (Figure 4).

DISCUSSION

IMT is a lesion with intermediate biological behavior that may recur with local or surrounding infiltration or, in rare cases, metastasis. IMT has been observed in almost all solid organs; however, its incidence in the liver has been low[1]. The case presented here involved a 42-year-old woman with a mass located on the right lobe of the liver; according to the pathological and immunohistochemical findings, the mass was diagnosed as IMT. One and a half years after surgery, no recurrence or metastasis have been identified.

This case contradicted the frequent male predilection of the lesion (male/female: 1.5/1), demonstrating that liver IMT was not limited to younger age groups and males[1]. Clinical findings indicate that symptoms in most cases include fever, abdominal pain, weight loss, weakness, and anorexia. Laboratory investigations usually suggest an inflammatory process: leukocytosis, neutrophilia, elevated ESR and CRP, anemia, thrombocytosis, polyclonal hypergammaglobulinemia, and slightly elevated levels of liver enzymes. Tumor markers, such as serum AFP and CEA, are always normal. Although these markers are valuable in the discrimination of liver IMT from malignant tumors, they cannot completely rule out some malignancies in which AFP levels may also be normal, such as fibrolamellar hepatocellular carcinoma. In this

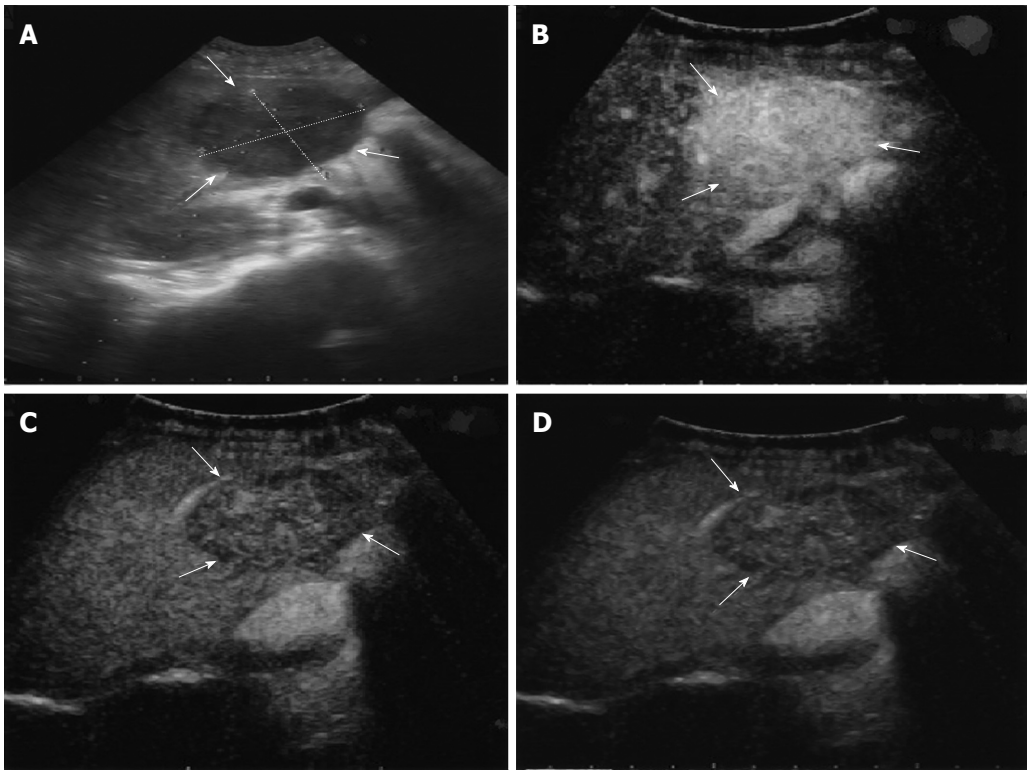


Figure 1 Ultrasonography images of the liver inflammatory myofibroblastic tumor. A: Conventional ultrasonography showing a 3.8 cm × 4.6 cm hypoechoic mass in the right anterior lobe of the liver, with a clearly defined border (white arrows); B: Arterial-phase images showing a hyperenhanced mass on the liver (white arrows); C: Portal venous-phase images showing slight enhancement and wash-out of the mass, which corresponded to that seen on arterial-phase images (white arrows); D: Parenchymal-phase images showing further wash-out of the mass, which corresponded to that on arterial-phase images (white arrows).

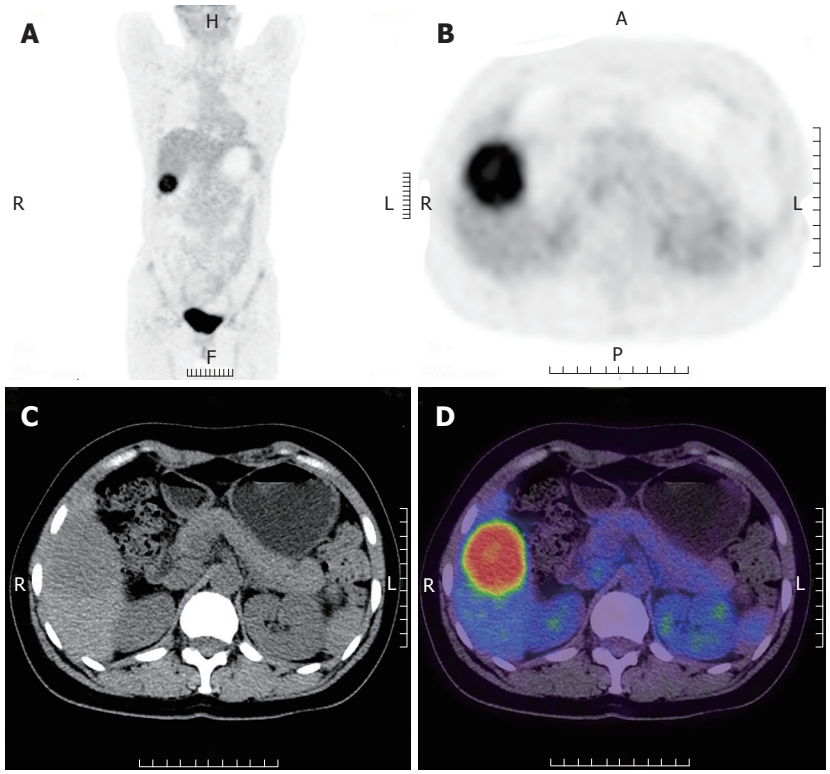


Figure 2 (Fluorine 18) fluoro-2-deoxy-D-glucose positron emission tomography and computed tomography images of the liver inflammatory myofibroblastic tumor. A: Coronal positron emission tomography (PET) demonstrating an intense (fluorine 18) fluoro-2-deoxy-D-glucose (^{18}F-FDG) uptake with SUV standardized uptake value in the anterior segment of right lobe of the liver; B: Axial PET demonstrating an inhomogeneous intense ^{18}F-FDG uptake; C: Axial computed tomography (CT) demonstrating a round mass with low density; D: Axial PET/CT demonstrating an inhomogeneous intense ^{18}F-FDG uptake in the anterior segment of right lobe. R: Right; L: Left; A: Anterior, P: Posterior; H: Head; F: Foot.

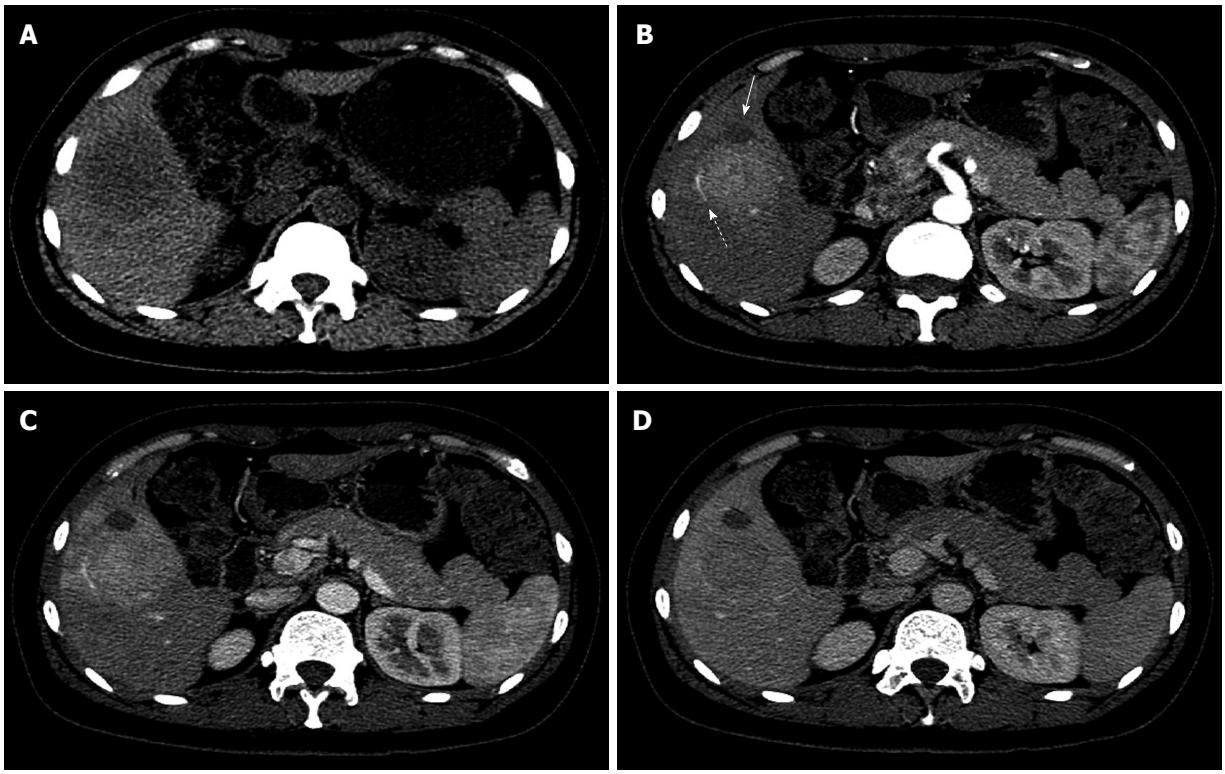


Figure 3 Axial contrast enhanced computed tomography images of the liver inflammatory myofibroblastic tumor. A: Axial arterial phase images showing a hyperenhanced mass well defined at the periphery of the mass; B: Arterial phase images showing a liver lobe artery (dash arrow) around the mass and the front edge of the mass lay a low density nodule (solid arrow) not enhancing in three phase images; C: Axial portal venous phase images showing a further enhanced corresponding to the arterial phase images; D: Axial delayed images showing obviously wash-out corresponding to the arterial phase images.

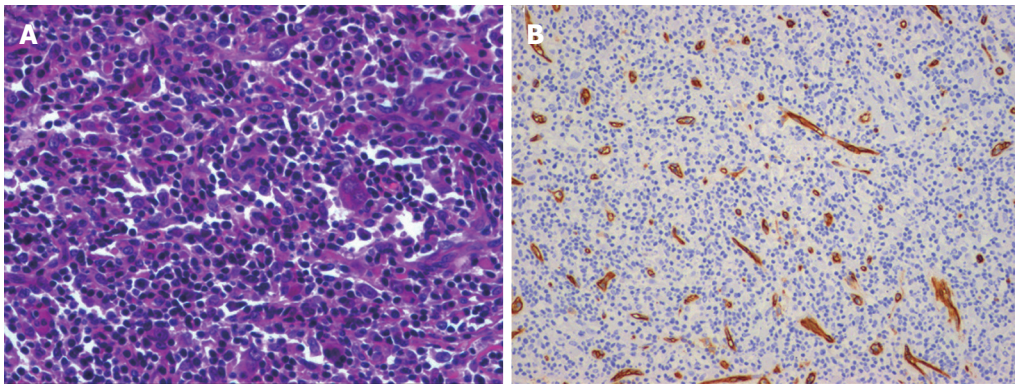


Figure 4 Histopathology findings of the liver inflammatory myofibroblastic tumor. A: Lesion was composed of chronic inflammatory cells and spindle cells (hematoxylin and eosin stain, × 200); B: Spindle cells were positive in smooth muscle actin tumor (immunostaining, × 100).

case, the clinical symptoms were nonspecific. The patient denied having abdominal pain, fatigue and weight loss. As described in the laboratory setting, the aminotransferases were all normal.

Furthermore, radiological aspects of IMT are usually similar to those of a malignant neoplasm[2]. Mali *et al*[3] reported a homogeneous mass without calcification or contrast enhancement. Megumi *et al*[4] reported that CECT revealed a soft, deeply stained image on the margin of the mass in the early phase. In the late phase, enhancement of the center of the mass was poor, and there was a soft, deeply stained image around the center. In this case, the CECT showed a hyperenhanced image in the arterial phase and a further developing mass in the portal venous phase; the contrast was obviously washed out in the delayed phase. On the front edge of the mass lay a low-density nodule, indicating the necrotic liver tissue that was removed by the surgeon, which has never been reported. However, the CECT image results of our case conflicted with the above case reports. These findings support the notion that the IMT CT image findings were nonspecific and could mimic many diseases.

Nam *et al*[5,6] observed two different appearances of the hepatic IMT. The enhancement was broad and ill-

defined at the periphery of the mass. In our case, the enhancement was broad and well defined at the periphery of the mass. In agreement with the histopathologic findings, the hypo-enhanced areas identified in the CECT correlated with the concentrated fibrous tissue, and the hyper-enhanced area corresponded predominantly with the cellular infiltration that contained foamy histiocytes, plasma cells, and lymphocytes. These image and pathological results corresponded to the findings in this study. To our knowledge, there has been no report on liver IMT with PET/CT. Wan *et al*[7] reported that inflammatory tissue has high metabolic activity. In addition, this case demonstrated an inhomogeneous, intense ^{18}F-FDG uptake with SUVmax 9.1. The inhomogeneous ^{18}F-FDG uptake of the mass corresponded to the different mass components (collagen fibers, chronic inflammatory cells, T-lymphocytes, ganglion-like cells).

IMT may be more difficult to clinically differentiate from malignant tumors, and different types of image findings and an excision biopsy may frequently be needed to make the diagnosis. The postoperative prognosis of liver IMT is favorable[4]. Some reports have shown that percutaneous needle biopsies are efficient for making the diagnosis[8]. When malignant tumors are suspected, a needle biopsy is not necessarily recommended because there is a risk of tumor cell dissemination. Because it may show spontaneous regression, indications for surgery must be carefully assessed. It is important that similar clinical cases perform ^{18}F-FDG PET/CT, CECT or MRI.

The pathogenesis of IMT is uncertain. Some authors have suggested an infectious origin; however, few cases have provided positive cultures or microorganisms isolated from tissue sections[2]. It was suggested that the chronic xanthogranulomatous pattern was a response to a previous infectious process. IMT can occur after surgery, trauma, radiotherapy or steroid use; it can also occur in connection with ventriculo-peritoneal shunts or infectious agents, such as *Mycobacterium avium intracellulare*, *Corynebacterium equi*, *Campylobacter jejuni*, *Bacillus sphaericus*, *Coxiella burnetii*, *Epstein-Barr* virus, *Escherichia coli*, *human herpes* virus 8 and *Actinomycosis*[3,9,10]. Some IMT cases are related to infectious or reparative processes, and the association of IMT with some infectious agents has been related to its pathogenesis, although it is rarely observed. According to some authors[6], IMT could develop secondary portal venous infection, and the inflammatory mass might enlarge together with the obliterative phlebitis, which may explain the tumoral development in the patient. As previously described in the literature, this tumor was found in the right lobe of the liver and was characterized by a well-circumscribed firm lesion with myxoid areas and a yellow or whitish color. Fleshy or myxoid surfaces suffering from lacerations rarely exhibit hemorrhage, necrosis or calcification[1]; however, this case showed necrotic liver tissue on the CECT and the CEUS, and this necrotic tissue was removed by our surgeon. The differential diagnosis depends upon the histological patterns observed; although we observed a hypocellular fibrous pattern with some inflammatory cells, the SMA immunoreactivity in the spindle cells was important in the diagnosis. CD34, CD23 and CD117 were non-immunoreactive in the spindle cells, which excluded the diagnosis of a fibrous solitary tumor. Although ganglion-like cells were positive in this case, there was no malignant tendency.

In this case, there was notable evidence of necrosis in front of the mass and a lobe artery at the periphery of the mass, which sometimes relates to malignant tumors. Although clinical, histopathological or molecular features do not predict biological behavior[10], recurrences are associated with the abdominal site, large size of the tumor and older age. Because the tumor may regress spontaneously, indications for surgery must be carefully assessed. Although ^{18}F-FDG PET/CT examinations can successfully differentiate IMT from other types of malignant tumors, the appropriate method of diagnosing liver IMT is still *via* histopathologic examination.

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CASE REPORT

Midgut volvulus due to jejunal diverticula: A case report

Jia-Li Hu, Wei-Zhong Chen

Jia-Li Hu, Wei-Zhong Chen, Department of Gastroenterology, Changzheng Hospital, Shanghai 200003, China
Author contributions: Hu JL wrote the manuscript; Chen WZ revised the paper.
Correspondence to: Dr. Jia-Li Hu, Department of Gastroenterology, Changzheng Hospital, No. 415 Fengyang Road, Shanghai 200003, China. shalley1@hotmail.com
Telephone: +86-21-81885344 Fax: +86-21-81885345
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Abstract

Jejunal diverticulosis is uncommon and often asymptomatic. It can produce significant complications, and some complications are potentially life threatening and require early surgical treatment, such as obstruction, hemorrhage and perforation. There is no consensus on the management of this disease. Only a few cases of jejunal diverticulosis with midgut volvulus have been reported. We herein report a case of 57-year-old woman with jejunal diverticulosis causing small bowel volvulus who complained of intermittent upper abdominal pin-prick for 5 years that eventually progressed to a complete obstruction. The computed tomography scans revealed a mesenteric vessel "whirlpool" and laparotomy showed midgut volvulus secondary to jejunal diverticula. This case highlights jejunal diverticulosis causing small bowel volvulus as an uncommon mechanism of small bowel obstruction, which should be included in the differential diagnosis of small bowel obstruction.

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Key words: Jejunal diverticulosis; Midgut volvulus; Intestinal obstruction; Computed tomography; Surgery

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INTRODUCTION

Although jejunal diverticulosis is rare and often asymptomatic[1], it can produce severe complications, such as obstruction, diverticulitis, hemorrhage and perforation. Obstruction can be caused by inflammatory stenosis, jejuna stones of the small bowel or small bowel volvulus. Most of the patients with gastrointestinal obstruction may require surgical intervention. Here we describe a patient with midgut volvulus due to jejunal diverticula. The computed tomography (CT) scans revealed a mesenteric vessel "whirlpool" and laparotomy showed midgut volvulus secondary to jejunal diverticula.

CASE REPORT

A 57-year old woman presented to our department with a 5-year history of increasingly intermittent upper abdominal pin-prick pain, which would occur after meals and eased in the right lateral decubitus. She denied any change in bowel or bladder habits, or presence of other gastrointestinal symptoms. There was no other relevant past medical, family or surgical histories. Left upper quadrant tenderness was found on physical examination.

The initial contrast-enhanced CT scan of the abdomen showed mesentery and superior mesenteric artery with "whirlpool" signs (Figure 1). However, most whirl signs exhibited on CT suggest but not prove to be indicative of small bowel volvulus[2]. Therefore, the patient was referred for an angiography due to the suspicion of a vascular variation as a reason to explain her intermittent upper abdominal pain. On the angiography, the superior mesenteric artery appeared twisted. Subsequent contrast barium study was performed with the suspicion of intestinal volvulus. The upper gastrointestinal barium study showed a diverticulum of duodenum, multiple gi-



Figure 1 Initial contrast-enhanced computed tomography scan of the abdomen showed mesentery and superior mesenteric artery with "whirlpool" signs (arrow).



Figure 2 Upper gastrointestinal barium studies demonstrated duodenal diverticulum (white arrow) and "corkscrew" configuration (black arrow) of proximal small bowel.



Figure 3 Upper gastrointestinal barium study showed multiple giant jejunal diverticula.



Figure 4 Extremely enlarged jejuna were seen after distortion. Enlargement starts 20 cm distal to the ligament of Treitz and extends to 120 cm.

ant jejunal diverticula and certified midgut volvulus (Figures 2 and 3).

On the fourth day of hospitalization, the patient experienced a worsening of pain. Physical examination revealed tenderness in the whole abdomen, with tinkling bowel sounds. Abdominal X-ray showed dilated bowel loops and air-fluid levels. Surgery was indicated based on the clinical manifestations, the symptoms of obstruction and the examination results.

She underwent an emergency laparotomy which revealed diffuse giant jejunal diverticulosis and midgut volvulus. The diameters of these diverticula ranged between 3.5 cm and 8 cm. The whole jejuna and its mesentery were found twisted clockwise by 540-degree at the root of mesentery. The proximal half of the small bowel was dilated, congested, and oedematous (Figure 4). Excision of the small bowel containing multiple jejunal diverticula and an end-to-end anastomosis of the jejuna were performed. The patient had an uneventful postoperative recovery and was discharged on the 12th post-operative day.

DISCUSSION

Jejunal diverticulosis is a rare entity with an incidence rate ranging from 0.3%-1.3% in autopsy series to 2.3% in radiographic findings[1]. Etiology of jejunal diverticulosis is still uncertain. Increased intraluminal pressure may play an important role. The mean age of symptomatic patients is up to 60 years in all reported studies. It is reported that jejunal diverticulosis associated with visceral neuropathies can lead to an increased intraluminal pressure, and it is commonly seen in connective tissue diseases, such as systemic lupus erythematosus[3-6].

About 80% of the jejunal diverticulosis cases present with no symptoms, and chronic clinical manifestations may be misdiagnosed as dyspepsia and irritable bowel syndrome, which delayed the correct diagnosis in its early phase.

A few symptomatic patients may explain of chronic post-prandial abdominal pain, nausea and vomiting, borborygmi, alternating diarrhea and constipation, weight loss, anemia, steatorrhea, tenderness and fever[7]. We should pay attention to these symptoms which reflect inflammation, malabsorption, hemorrhage or mechanical obstruction.

Mechanical intestinal obstruction occurs in 2.3%-4.6% of cases of jejuno-ileal diverticulosis[8]. Obstruction can be caused by inflammatory stenosis, intussusceptions and voluminous jejuna stones. Besides, in jejuno-ileal

diverticulosis, volvulus of the diverticula-containing segment is a cause of intestinal obstruction. The involved segment, which is filled with fluid and is heavier than the non-involved part, might be responsible for initiating the volvulus[8]. It is reported that a large small-bowel diverticulum was much more frequently seen in patients with volvulus (35%) than in those with small bowel volvulus (1%) ($P < 0.001$)[9].

The diagnostic work-up in asymptomatic patients can start with plain abdominal X-ray or CT. But in some cases, these modalities fail to show typical jejunal diverticula. Since they are situated along the mesenteric border, it makes the diagnosis difficult. The specific diagnosis of small bowel diverticula is possible by radiologic contrast studies using various forms of barium[10,11]. The radiologic technique of enteroclysis is specific for the jejuna and the ileum, and the barium meal for the duodenum[12]. Similar to the diverticulitis of the large intestine, perforation is the most common complication in diverticulitis of the small intestine[13]. Perforation is associated with peritonitis in most cases. Thus, we should choose iodine contrast instead of barium if perforation is suspected, considering the high mortality of barium solution peritonitis. Enteroscopy may be another feasible method for diagnosing small bowel diverticulosis[10,14]. In recent years, endoscopy has replaced enteroclysis and has become the most widespread technique for the diagnosis of small bowel diverticula[15].

In case of acute abdomen pain due to small bowel volvulus, repeated physical examinations and X-ray films are recommended. Besides, CT examination and angiographic study are helpful in diagnosis. The "whirl sign" on abdominal CT refers to a whirling or spiral shape of the mesenteric vessels, which may accompany the intestinal loops and their feeding vessels[2,16]. Fisher first described it as a CT finding suggestive of midgut volvulus[17]. It is a sensitive marker of volvulus with a sensitivity of 60% and an odds ratio of 25.3 in predicting the presence of small bowel obstruction necessitating surgery[18]. Awareness of the CT whirl sign is important in the triage of patients who need emergency surgical intervention[19]. Although angiography is not feasible for all the suspected cases of intestinal volvulus due to its invasive nature and high cost, it is still a good method for preoperative evaluation of the "whirlpool" and "barber's pole"[16].

Usually uncomplicated jejunal diverticulosis does not require operative management. However, surgical intervention is mandatory in patients with complications of jejunal diverticulosis. Surgical therapy of jejunal diverticulosis with small bowel volvulus consists of untwisting or resecting the involved segment. A mortality rate of 25% was reported[8].

In our case, the main clinical symptom in the patient's history was increasingly intermittent upper abdominal pin-prick pain with vomiting. It was caused by hyperdistention of the voluminous diverticula. The thickened and enlarged small bowel found at laparotomy would suggest that partial obstruction was present for some time before the acute episode.

In summary, we present a case of a midgut volvulus which was induced by jejunal diverticula. Jejunal diverticulosis remains asymptomatic in about 80% of cases, making the diagnosis both delayed and difficult. Diagnosis is often initiated in cases with related complications, such as hemorrhage, perforation or obstruction. Intestinal obstruction can be caused by inflammatory stenosis, jejunal stones, intussusception or volvulus. It is suggested that a large small-bowel diverticulum might play a contributing role in the occurrence of small bowel volvulus in adults. Enteroclysis is probably the most reliable method for diagnosing small bowel diverticulosis. In case of acute abdomen pain due to small bowel volvulus, CT examination shows typical signs of this disorder. The whirl signs detected on CT have a high specificity for small bowel volvulus. Surgical excision of the affected jejuna segment with primary end-to-end anastomosis is the treatment of choice.

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CASE REPORT

Primary squamous cell carcinoma of the liver associated with hepatolithiasis: A case report

Ke-Lei Zhu, Ding-Yao Li, Cun-Bing Jiang

Ke-Lei Zhu, Ding-Yao Li, Cun-Bing Jiang, Department of Hepatopancreatobiliary Surgery, Yin Zhou People's Hospital, Ningbo 315040, Zhejiang Province, China

Author contributions: All authors contributed equally to this work; Zhu KL designed the research and wrote the manuscript; Li DY and Jiang CB participated in the discussion of the clinical and pathological features of the case.

Correspondence to: Ke-Lei Zhu, MM, Department of Hepatopancreatobiliary Surgery, Yin Zhou People's Hospital, Ningbo 315040, Zhejiang Province, China. dr.zkl@163.com
Telephone: +86-574-87016881 Fax: +86-574-87017272
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Abstract

Primary squamous cell carcinoma (SCC) of the liver is rare and reported sporadically. Up to date, only 24 such cases have been reported in the literature. It is associated with hepatic teratoma, congenital cysts, solitary benign non-parasitic hepatic cysts, hepatolithiasis/Caroli's disease or cirrhosis. We reported a case of primary SCC of the liver associated with multiple intrahepatic cholesterol gallstones. The patient underwent hepatectomy followed by radiotherapy, and has survived for over 19 mo without recurrence.

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Key words: Hepatolithiasis; Surgery; Radiotherapy; Squamous cell carcinoma

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INTRODUCTION

Primary squamous cell carcinoma (SCC) of the liver is rare and reported sporadically. Only 24 such cases have been reported in the literature. It is found to be associated with hepatic teratoma[1], congenital cysts[2-14], solitary benign non-parasitic hepatic cysts (SBNHC)[2-6,8-11], hepatolithiasis/Caroli's disease[15,16] or cirrhosis[17]. The carcinogenesis seems to be related to the chronic inflammation of the biliary epithelium or the metaplastic and subsequent neoplastic transformation of cysts of the liver[15]. Although various therapeutic strategies, including systemic chemotherapy, radiotherapy, transarterial chemoembolization and hepatic resection, have been used, the prognosis of primary SCC of the liver is dismal with an overall survival less than one year.

We report a case of primary SCC of the liver occurring in association with hepatolithiasis. The patient underwent hepatectomy in January 2010 followed by radiotherapy, and has survived for over 19 mo without recurrence.

CASE REPORT

A 46-year-old woman presented with right upper quadrant pain and a 15-pound weight loss over the past five weeks before admission in January 2010. There was no radiating pain, vomiting, jaundice, fever, chill, cough or irregular vaginal bleeding. She had a history of hepatitis A, but without a history of alcohol drinking, smoking or surgery. On physical examination, the abdomen was soft, and no tenderness, rebound tenderness and palpable abdominal mass were found. The biochemical data were as follows: hemoglobin 110 g/L, white cell count 5.2 $\times 10^9$/L with neutrophil 82.5%, platelet 334 $\times 10^9$/L, alanine aminotransferase 28 μ/L, serum total protein 70 g/L, serum albumin 34.1 g/L, serum total bilirubin 15 μmol/L, serum direct bilirubin 9.5 μmol/L, alpha-fetal protein 7.33 ng/mL, and carcinoembryonic antigen (CEA) 8.5 ng/L, and serologic markers for the hepatitis-B virus were not detected. The plasma retention rate



Figure 1 Computed tomography scan showed a regular mass with inhomogeneous density, mild delayed enhancement in the peripheral zone, and necrosis in the central zone. R: Right.

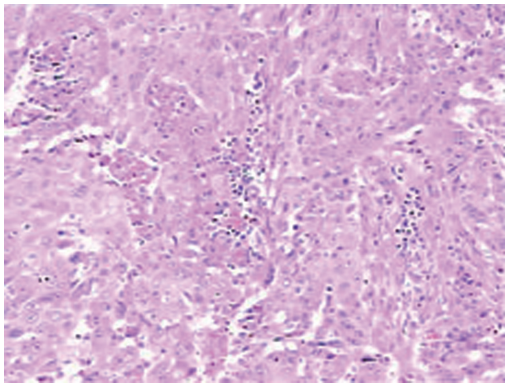


Figure 3 Histology of the tumor: Squamous cells with keratinization and areas of necrosis. Hematoxylin-eosin stain, × 10.

of indocyanine green at 15 min was 1.2%. Subsequent abdominal ultrasonography showed a mixed echoic mass measuring about 5.0 cm × 6.0 cm occupying the right lobe of the liver. The abdominal computed tomography (CT) showed a regular mass measuring about 5 cm × 6 cm at its greatest dimension with inhomogeneous density, mild delayed enhancement in the peripheral zone, necrosis in the central zone of the tumor and dilated secondary biliary ducts with intraductal lithiasis (Figures 1 and 2). Gastrointestinal endoscopy and colonoscopy showed negative findings. Chest CT revealed no mass over the lung.

The initial diagnosis before operation was intrahepatic cholangiocarcinoma (ICC) according to the CT findings (a regular mass, periductal dilatation and intraductal lithiasis). Surgery remains the exclusive choice of curative therapy for ICC. The patients underwent right hepatectomy and cholecystectomy in February 2011. A hard and yellowish white tumor mass was found, measuring about 5 cm × 6 cm at the greatest dimension with central necrosis occupying mainly S5 of a non-cirrhotic liver and numerous biliary calculi in the dilated intrahepatic bile duct adjacent to the tumor. The specimen appeared with a 1-cm negative margin without cysts on its cut surface.

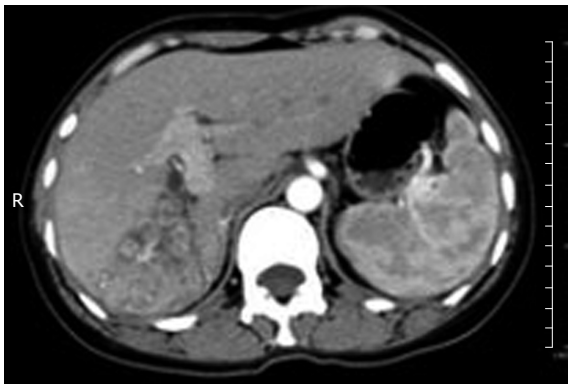


Figure 2 The bile ducts adjacent to the tumor were obviously dilated and full of biliary calculi. R: Right.

Histopathological examination showed a poorly-moderately differentiated SCC composed of squamous cells with keratinization and large areas of necrosis (Figure 3). The secondary bile ducts adjacent to the tumor were obviously dilated and full of biliary calculi. Chronic inflammatory lesions were found in the biliary epithelial and gallbladder mucosa without evidence of squamous metaplasia. Immunohistochemical tests were positive for cytokeratin (CK) 14, CK19 and CEA, but negative for CK 18 and thyroid transcription factor 1 (TTF-1). CK 14 positive indicated that basal cells of keratinized squamous epithelium were originated from the cancer cells. Metastatic lung or thyroid cancer to the liver could be ruled out, as TTF-1, an indicator of small cell carcinoma of the lung or thyroid, was negative. Subsequently a thorough search for a primary tumor showed a negative finding in the oral cavity and nasopharynx and a mild inflammatory change in cervical smear. Taken together, the final diagnosis of primary SCC of the liver was made.

The post-operative course was uneventful and the patient was discharged on the 14th day after operation. The patient was started on radiotherapy 2 mo after operation. Routine follow-up was continued in the hepatobiliary clinic. No tumor recurrence and distant metastasis were found during the 19-mo follow-up period.

DISCUSSION

Primary SCC of the liver is very rare, and only 25 cases have been reported from 1934 till now (including the present one) in the literature. Among 13 (52%) of these cases, SCC arose from congenital hepatic cysts[2-14] or SBNHC[2-6,8-11]. There were only two cases of SCC associated with hepatolithiasis/Caroli's disease[15,16]. The other cases were associated with cirrhosis and teratoma[1,17]. The precise steps leading to the carcinomas are unclear. Positive CK14 and negative CK18 indicate that basal cells of keratinized squamous epithelium are originated from the cancer. Positive CK19 indicates that the tumors are transformed from chronic inflammation of the biliary epithelium. Thus, chronic inflammation of the biliary epithelium might be the most reasonable mechanism

of carcinogenesis.

Clinically, patient presents with the abdominal pain and weight loss as initial symptoms. CT scan of the abdomen is helpful for evaluating the primary SCC, such as the number of lesions, location, extension, and whether the tumor invades surrounding structures. If the mass is suspected to be malignant, biopsy or even surgical resection should be conducted if patient's condition permits.

Although several management strategies have been proposed, ranging from hepatectomy, systemic chemotherapy and radiotherapy to hepatic arterial injection of low-dose anti-cancer drugs, the prognosis of primary SCC of the liver is dismal with an overall survival less than one year. Naik *et al*[18] described a patient with SCC who survived for over 6 year after preoperative radiotherapy and surgical resection. Boscolo *et al*[19] reported a case of complete remission of poorly differentiated SCC after systemic chemotherapy (cisplatin + 5-fluorouracil) and surgery. Spaggiari *et al*[16] reported a case with a 7-year survival after right hepatectomy. In our case, SCC occurred in association with hepatolithiasis. The patient was successfully treated by hepatectomy followed by radiotheraphy. The postoperative course was smooth, and she is still alive 19 mo after the operation without recurrence.

In conclusion, we described the first case of primary SCC of the liver associated with hepatolithiasis. The patient was successfully treated by surgery followed by radiotherapy. She has survived for 19 mo with no tumor recurrence and distant metastasis.

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Yasuhiro Fujino, MD, PhD, Director, Department of Surgery, Hyogo Cancer Center, 13-70 Kitaoji-cho, Akashi 673-8558, Japan

Mark D Gorrell, PhD, Professor, Centenary Institute of Cancer Medicine and Cell Biology, Locked bag No. 6, Newtown, NSW 2042, Australia

Po-Shiuan Hsieh, MD, PhD, Head of Department of Physiology and Biophysics, National Defense Medical Center, Taipei 114, Taiwan, China

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Atsushi Tanaka, MD, PhD, Associate Professor, Department of Medicine, Teikyo University School of Medicine, 2-11-1, Kaga, Itabashi-ku, Tokyo 173-8605, Japan

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Dr. Pingchang Yang, MD, PhD, Department of Pahtology and Molecular Medicine, McMaster University, BBI-T3330, 50 Charlton Ave East, Hamilton L8N 4A6, Canada

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

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American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

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Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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